IMPROVEMENT OF AN ADENOVIRUS-BASED GENE VECTOR: SYNTHETIC MATERIALS THAT REDUCE IMMUNOGENICITY AND FUNCTION IN PLACE OF THE FIBER PROTEIN

By

ADANE SEBSIBE NIGATU

Bachelor of Science in Chemical Engineering
Bahir Dar University
Bahir Dar, Ethiopia
2006

Master of Science in Chemical Engineering
Oklahoma State University
Stillwater, Oklahoma, USA
2012

Submitted to the Faculty of the
Graduate College of
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2015

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Dissertation Approved:

Dr. Joshua D. Ramsey

Dissertation Adviser

Dr. R. Russell Rhinehart

Dr. Heather Fahlenkamp

Dr. Babu Fathepure

ACKNOWLEDGEMENTS

I take this opportunity to thank all people who have helped me produce this dissertation. First, I would like to express my sincere gratitude to my adviser Dr. Joshua D. Ramsey for his consistent guidance and support during the course of my studies and research. It has been a pleasure to work with and for him. I would also like to thank members of my research committee, Dr. Fahlenkamp, Dr. Rhinehart and Dr. Fathepure for their advice. I thank my lab mates for all the help they have given me.

My mother, who passed away recently, has given a lot to see me and my siblings succeed in life. I am greatly indebted to her. Her endless support and encouragement made me the person I am. Her prayers has kept me safe and blessed. Looking at me for the heavens, I hope I made her proud.

I am also grateful to my father.

My brother Yonas and my sisters Misrak, Tsion, Eyersalem, and Eden have supported me in any way they can. Memories of my sister Hirut and brother Himanot, who passed away at my younger age, have kept me grounded.

My wonderful wife Fikirte (Kemem) who is the driving force to my life has supported me to accomplish a lot. I'm forever grateful for her endless love and patience.

It is impossible to thank everyone that helped me along the way. I am very grateful and would like to thank all my friends here in the US and back in Ethiopia for supporting me during my transition to the United States to pursue my graduate education.

Finally, I would like to thank Oklahoma State University School of Chemical Engineering for the rich learning experience.

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CHAPTER I

INTRODUCTION

1.1. Gene Therapy

In the course of hundreds of years of pursuit to understand the driving force of life, the scientific community discovered DNA: the source of information to build and maintain an organism. In the 1860's Friedrich Miescher discovered nuclein, a term which eventually was changed to deoxyribonucleic acid (DNA)(Dahm 2005). Phoebus Levene and later Erwin Chargaff uncovered the components of DNA and how the molecules are put together (Levene 1920, Chargaff, Zamenhof et al. 1950). Based on these previous works, Francis Crick and James Watson, with the help of Rosalyn Franklin and Maurice Wilkins, identified the structure of DNA (Watson and Crick 1953). The Human Genome Project, an international scientific research project, advanced the understanding of the genetic code embedded in the DNA by identifying the genetic makeup and mapping human DNA (Sedlak 2003).

Progress in understanding human DNA made it clear that abnormalities in the genome can lead to genetic malfunctions and are the cause of many diseases. In 1970, Stanfield Rogers

proposed the use of "good" DNA to replace inherited malfunctioning DNA as a treatment for diseases caused by abnormal genes (Friedmann 2001). Later this proposal led to gene therapy, the treatment of diseases by correcting defective genes (Mountain 2000, Nathwani, Benjamin et al. 2004). Gene therapy has promising potential to treat acquired diseases such as cancer (McNeish, Bell et al. 2004) cardiovascular diseases (Katz, Swain et al. 2010), neurological diseases (Ribotta 2001), and inherited diseases such as muscular dystrophy (Inui, Okada et al. 1996) and cystic fibrosis (Griesenbach, Geddes et al. 2006).

The first gene therapy clinical trial was conducted in 1990 by French Anderson and colleagues at the NIH. The study was conducted to cure two children with Adenosine deaminase (ADA) deficiency that damages the immune system (Sheridan 2011). The trial involved the transfer of gene encoding ADA into T-cells extracted from the patient. The T-cells were then administered back to the patient post treatment. After the clinical trial, one patient exhibited a temporary response and was further treated with enzyme replacement therapy. Even though the treatment did not totally reversed the phenotype, this study is considered a proof-of-concept for gene therapy. Since then, over 2000 human gene therapy clinical trials have been conducted (Edelstein 2014).

The first successful gene therapy to provide full correction of the diseased phenotype was conducted in 2000 (Cavazzana-Calvo, Hacein-Bey et al. 2000). Two children with X-SCID, an X-linked inherited immunodeficiency disorder caused by the inability of T- and B-lymphocyte to differentiate, were treated with murine leukemia virus carrying complimentary DNA that encoded a cytokine receptor essential in promoting differentiation and growth signals to progenitor cells. Although the treatment was successful in correcting the disorder, it was later found that five out of 20 patients who

received the treatment developed leukemia (Hollon 2000). Studies suggested that the development of leukemia was attributed to retroviral gene insertion to host cell genome (Couzin and Kaiser 2005). This incident raised a safety concern. The practicality of gene therapy was further questioned when a patient with ornithine transcarbamylase deficiency died after being treated with an adenovirus gene vector (Raper, Chirmule et al. 2003). The cause of death was later associated with an immune response of the patient to the dosages of the vector used for the gene delivery (Hollon 2000). These events suggested that a better understanding of the delivery mechanism is vital for a safe and effective application of gene therapy (Orkin 1996, Verma and Somia 1997, Zaia 2007).

Depending upon the type of disease, the therapeutic gene, and the gene delivery vector, gene therapy can be *in vivo* or *ex vivo* (Fig 1.1). An *in vivo* gene therapy involves the administration of the therapeutic gene into the patient with the help of a delivery system which consists of a gene delivery vector. In this method, once the delivery system is developed, it can be used for all patients with similar diagnosis. *In vivo* gene therapy, however, usually causes the wide distribution of vectors to undesired tissues and leads to complications (Mountain 2000). *Ex vivo* gene therapy, in contrast, involves administration of treated cells originally extracted from a patient. The cells, then introduce the desired therapeutic change in the body of the patient. This method requires patient-specific tailoring, hence incurring high manufacturing costs and quality-control difficulties.

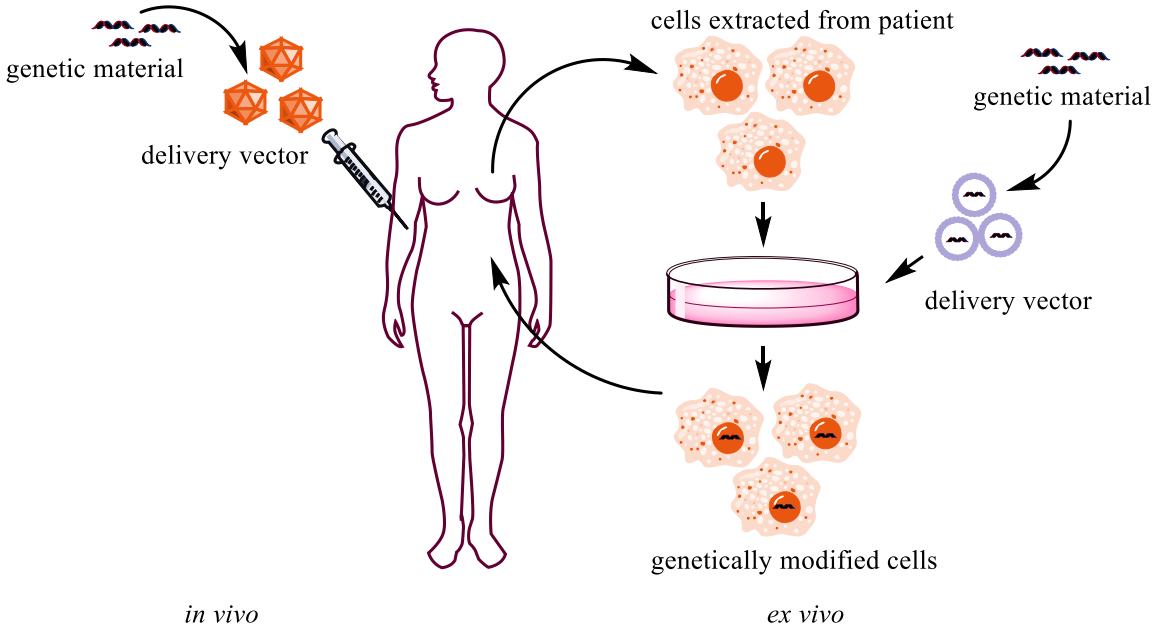

Figure 1.1: Schematic of *in vivo* and *ex vivo* gene therapy. *In vivo* gene therapy involves administration of the gene delivery vector that contains the therapeutic gene that helps treat the disease. *Ex vivo* gene therapy involves the use of cells extracted from the patient to provide the desired therapeutic change.

As mentioned earlier, *in vivo* gene therapy allows the treatment of multiple patients with similar conditions. This method, however, needs vectors that can maneuver through the patient's body to deliver the therapeutic gene to a specific tissue. Gene delivery vectors have to overcome tissue matrices, avoid enzymatic degradation, and evade inactivation by the immune system to effectively transfer the gene into the desired cell (Lechardeur, Sohn et al. 1999).

The vector has to first overcome extracellular barriers before reaching the surface of the target cell. The type of extracellular barrier depends on what kind of tissue is being targeted by the vector. For example, to target respiratory epithelium, the vector has to overcome extracellular barriers such as mucus, and other immune surveillance that prevents

penetration of the epithelial cells (Ferrari, Geddes et al. 2002). After passing the extracellular matrix, the vector has to bind to the cell surface of the target cell through specific cellular receptors which facilitate recognition and internalization (Wu, Wilson et al. 1989) (Fig 1.2). These steps, however, can be compromised by deficiency of cellular receptors or low cellular uptake of the target cell (Kay, Glorioso et al. 2001).

In the presence of the specific cellular receptors and efficient cellular uptake, the vector, in most cases, internalizes into cell through endocytosis (Cho, Kim et al. 2003). The vector has to transport through the endolysosomal network and escape the endosome at a location near the nucleus before being degraded by the harsh intracellular environment (Luzio, Mullock et al. 2001). The genetic material is then unloaded from the vector inside or at close proximity to the nucleus. The genes unloaded outside the nucleus have to inertly diffuse through the nuclear pore to enter the nucleus. Weakness of the nucleus membrane during mitosis can also promote diffusion of the gene into the nucleus for gene transcription (Wilke, Fortunati et al. 1996). Throughout these processes, the vector has to overcome intracellular harsh pH and degradative enzymes.

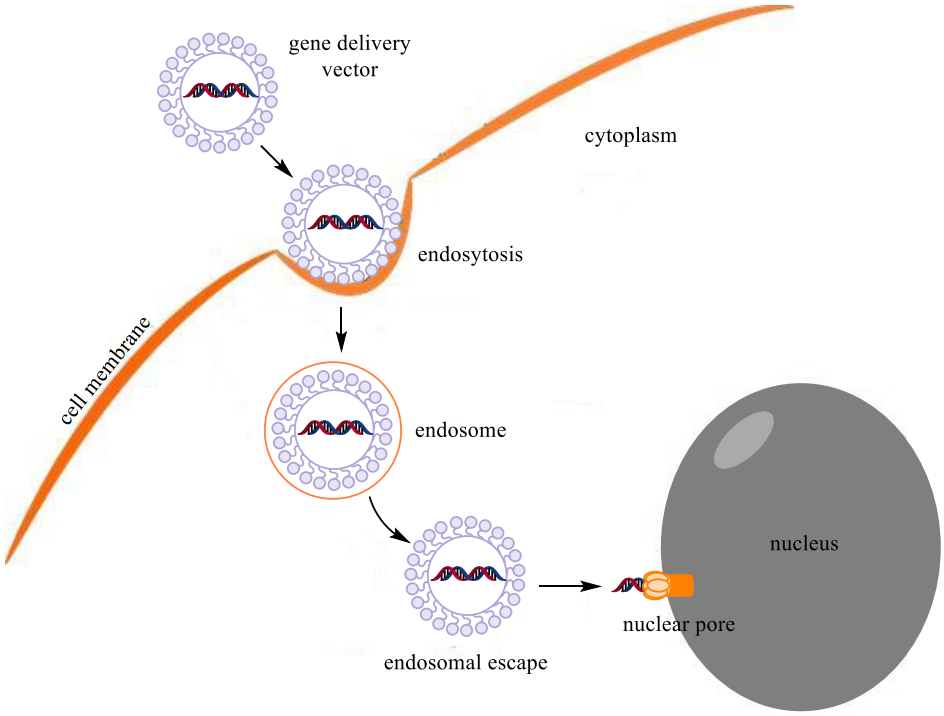

Figure: 1.2: Schematic diagrams of gene transport by gene delivery vector. The vector has to first bind to cellular components that induce endocytosis. After entering the cell, the vector has to escape the endosome and release the genetic material in close proximity *to* the nucleus. The genetic material is then taken up by the nucleus via the nuclear pore.

1.2. Gene Delivery Vectors

For gene therapy to progress beyond the lab, a safe and efficient delivery vehicle capable of carrying a gene to the desired tissue has to be developed. Ideally, gene delivery vectors have to be immunologically inert. Innate immune responses toward the vector induce the development of adoptive immunity and promote elimination of transduced target cells, while pre-existing humoral immune responses neutralize re-administrated vectors (Walther and Stein 2000). The cells targeted for gene delivery are usually found in a heterogeneous surrounding or assorted within different parts of the body. Gene delivery vectors, therefore, have to selectively deliver the genetic material to the target cells. Depending on the type of disease, gene expression can be transient, regulated, or life-time. For example, in cases

of vaccines, transient gene expression is required, while to treat diabetes, regulated gene expression is preferred. For diseases such as hemophilia, however, life-long gene expression is required to cure the disease. The vector should also house large-sized genetic materials for delivery protection. Another important property is that gene delivery vectors should not interfere with the replication, segregation or integration of therapeutic genes in the host cell. The therapeutic gene should reside as an episome or integrate into the chromosome in a site-specific manner. Unsought gene integration to the host chromosome might result in mutagenesis. Developing gene delivery vectors satisfying all these conditions has been very difficult (Somia and Verma 2000). In fact, gene delivery vectors are considered the 'Achilles heel' of gene therapy.

Gene delivery vectors are divided into viral and non-viral vectors. Viruses are an obligate intracellular parasite, capable of introducing their own genes into cells to multiply. Replication defective viruses are often used as vehicles to carry therapeutic genes into cells instead of their own gene. Viral vectors have high potential as they are extremely efficient and provide stable gene expression. In fact, 70% of gene therapy clinical trials used viral vectors (Edelstein 2014). Out of the viral vectors, adenovirus is used in 23% of gene therapy clinical trials, followed by retrovirus.

Unfortunately, viral vectors come with drawbacks. Issues surrounding oncogenicity (Kohn, Sadelain et al. 2003) and immunogenicity (Hollon 2000) have resulted in severe setbacks. Non-viral vectors such as naked DNA and liposomes, in contrast, are relatively safe. These types of vectors are likely to present lower immunogenicity and toxicity but lack the necessary effectiveness to deliver genes. Furthermore, non-viral vectors only provide a transient gene expression. A continued and high-level of gene expression is, however, often

required to treat diseases. Viral vectors, in this regard, are suitable vehicles to efficiently transfer therapeutic genes.

As mentioned above, adenovirus is one of the most widely used vectors in gene therapy clinical trials. Adenovirus can accommodate large transgenes and has the ability to transduce both dividing and non-dividing cells (Benihoud, Yeh et al. 1999, Mizuguchi and Hayakawa 2004). Adenovirus is also safe in the sense that it does not promote gene integration into the host chromosome, which leads to oncogeniesis (Couzin and Kaiser 2005). While adenovirus is relatively safe and has been widely used as a gene delivery vector, major drawbacks such as immunogenicity and promiscuous tropism are significant concerns. Adenovirus fiber and capsid proteins mediate an inflammatory response and initiate clearance of the virus in patients with preexisting immunity (Kafri, Morgan et al. 1998, Schagen, Ossevoort et al. 2004). The adenovirus infection pathway, however, is dependent on the binding interaction of the fiber proteins of the virus with coxsackievirus and adenovirus receptor (CAR) present on the cell membrane (Yang, Li et al. 1995, Bewley, Springer et al. 1999, Chirmule, Propert et al. 1999). The ubiquitous presence of this receptor in a wide range of tissues limits the use of adenovirus for targeted delivery. At the same time, the dependency of the virus on CAR compromises the ability of the virus to act as a gene transfer vector into cells such as advanced tumor cells, vascular smooth muscle cells, peripheral blood cells, dendritic cells, and hematopoietic stem cells that lack CAR (Wickham 2000, Mizuguchi and Hayakawa 2004, Campos and Barry 2007). Further, the virus shows promiscuous tropism toward other types of cells due to the secondary affinity interaction between cellular elements, such as integrin and proteoglycan, and motifs present on the fiber and capsid proteins of the virus, such as RGD (Wickham,

Mathias et al. 1993, Smith, Idamakanti et al. 2003). Clearly, adenovirus requires significant improvements to overcome these limitations before it can be considered an ideal gene delivery vector.

Chemical and genetic modifications of adenovirus have helped improve drawbacks associated with the virus. Chemical modification of adenovirus with polyethylene glycol (PEG) has been widely applied to improve undesired properties of the virus (O'Riordan, Lachapelle et al. 1999, Campos and Barry 2007). Genetic modification of the fiber protein of adenovirus leads to reduced natural tropism and a lessened immune response (Kirby, Davison et al. 1999, Legrand, Spehner et al. 1999, Kurachi, Koizumi et al. 2008). Chemical and genetic modifications, however, hinder the interaction of the fiber protein and receptor. To eliminate concerns associated with this protein, our group has developed a recombinant adenovirus with and without the viral fiber protein. Although the modification reduces the efficiency of the virus to transform cells, our previous studies and studies by others, have shown that the use of cell-penetrating peptide (CPP) and targeting ligands can address issues of reduced transduction and targeted delivery (Wickham 2000, Eto, Yoshioka et al. 2009, Nigatu, Vupputuri et al. 2013). *The working hypothesis of this study was twofold: (i) a hybrid vector developed from adenovirus and a CPP-polymer conjugate can maintain high transduction efficiency and reduced susceptibility of the virus to immune responses, and (ii) CPP-polymer and ligand-polymer conjugate can replace the function of the fiber protein to augment transduction and retarget the virus to specific cellular receptors and reduce vulnerability of the virus to immune responses.*

The goal of this study was to produce a safe and efficient gene delivery through chemical and genetic modifications and extend our understanding of transition to producing effective

synthetic vectors. The following objectives were met in the process of accomplishing this goal of the study (1) reduced susceptibility of native adenovirus to immune response and augmented gene delivery efficiency; and (2) replaced the fiber proteins of the virus with CPP-PEG and ligand-PEG, and produced a hybrid vector that is resistant to immune responses and possesses targeted delivery efficiency.

Ultimately synthetic vectors that accurately mimic a virus and deliver genetic materials without the dangers currently associated with viral gene delivery vectors must be developed. This study will function as a platform to better understand elements of vector development and assist in the initial stages of a stepwise transition from viral to synthetic vectors.

CHAPTER II

REVIEW OF LITERATURE

2.1 Adenovirus

Adenovirus, a member of the *Adenoviridae* family, was initially isolated from human adenoid tissues in 1953 (Rowe, Huebner et al. 1953). Adenoviruses have a propensity to produce disease such as acute respiratory diseases, urinary tract, and gastrointestinal infections. Currently, 57 adenovirus serotypes have been isolated based on their susceptibility to neutralizing antisera (Martin 2007). These adenovirus serotypes are further categorized into seven subgroups (A to G) based on their tendency to hemagglutinate. The next two section discuss the biology of adenovirus serotype 5.

2.1.1 Structure of Adenovirus

Adenovirus is a medium sized (150 MDa), icosahedral shaped, non-enveloped virus with an approximate diameter of 90 nm. Adenovirus has 36 kbp, non-segmented linear double-stranded DNA molecules. Cryo EM and X ray crystallography of the virus have shown that capsid of adenovirus is composed of 20 hexon capsid faces, 12 penton-base proteins, and 12 spike or fiber proteins emanating from each vertices (Rux and Burnett 2004). Capsid faces of the icosahedral shaped virus are made up of 720 hexons arranged in trimers with12

hexons making up each face. The hexons are glued to each other with adhesive protein IX (pIX) (Furcinitti, van Oostrum et al. 1989). Penton base sits on the 12 vertices of the structure. The fibers, a trimer protein, protrude from each penton-base protein (Fig. 2.1). The fiber protein is composed of three distinct regions: tails, a shaft domain, and a knob. Fiber proteins bind to cell surface receptors to facilitate uptake (Zubieta, Schoehn et al. 2005). The tails are composed of residues from the amino (N)-terminus, which attaches the fiber to the capsid penton base. The shaft consists of a trimer with 22 structural repeats connecting the tail and knob. The knob is a (C)-terminal segment that contains a receptor-binding domain and a trimerization domain (Medina-Kauwe 2003).

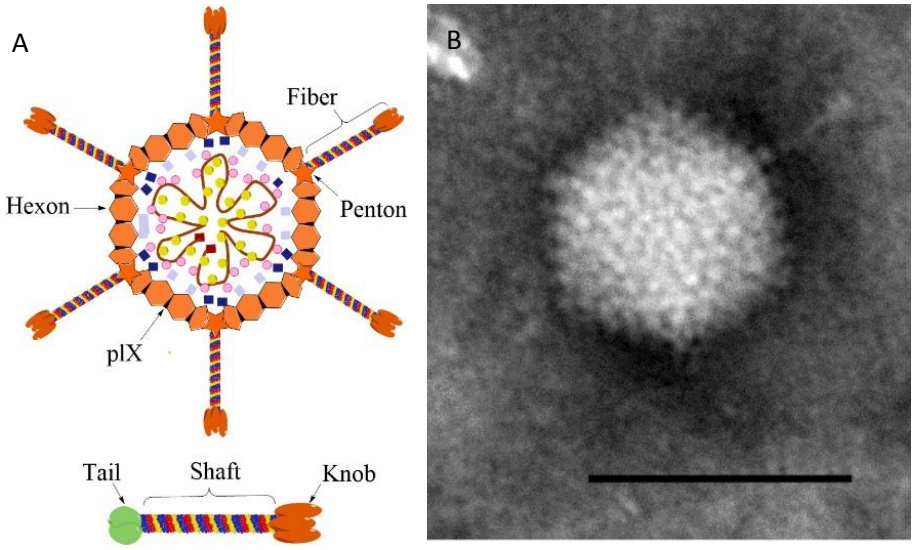

Figure 2.1: Structure of adenovirus. A) Depiction of adenovirus capsid protein (Hexon, Penton, and pIX) and components of fiber protein emanate out of the surface of the capsid. B) Transmission Electron microscopy image of adenovirus produced by our lab.

2.1.2 Generations of Adenovirus

Adenovirus vectors are grouped into three generations based on gene modifications (McConnell and Imperiale 2004). The first generation vectors are produced by removing

the early transcription units E1 and/or E3. E1 gene deletion produces a replication deficient virus. E3 gene encodes proteins necessary to protect cells from the immune system. The second generation vectors are produced by deleting E2 and E4 genes in addition to E1 and E3 genes. Complimentary cells such as the 293 that possess E1 genes are used to amplify the first and second generation virus. The third, the helper-dependent generation are developed by completely deleting all viral genomes except the ITR and the capsid signal. The first and second generation vectors trigger an immunity, while the helper-dependent generation has a reduced cellular immunogenicity (Imler 1995, Tatsis and Ertl 2004).

2.1.3 Infection Pathway of Adenovirus

The adenovirus infection pathway involves two distinct steps (Fig. 2.2). First, the fiber protein of the virus binds to receptors on the cell membrane. The knob domain of the fiber protein adheres to coxsackie B virus and the adenovirus receptor (CAR) and trimerizes the receptor (Howitt, Anderson et al. 2003). Other receptors such as heparin sulphate proteoglycans also play important role in linking the virus with the cell (Smith, Idamakanti et al. 2003). Next, the penton base interacts with cellular integrin receptors through the argentine-glycine-aspartic acid (RGD) motif (Wickham, Mathias et al. 1993). These interactions lead to receptor-mediated endocytosis of the virus through clathrin-coated vesicles (Meier and Greber 2003). After endocytic uptake, the virus encapsulated with an endosomal membrane, travels through a slightly acidic intracellular compartment. The condition stimulates the permeabilization of the endosomal membrane, promotes conformational change and partially disassembles capsid proteins (Graham, Smiley et al. 1977, Seth, Willingham et al. 1985, Furcinitti, van Oostrum et al. 1989, Greber, Willetts et al. 1993). After escaping the endosome, the virus particles travel to the nucleus along the

microtubules by binding with the dynein/dynactin motor complexes, which guide the particles to the nucleus (Kelkar, Pfister et al. 2004). Upon arriving near the nucleus, virus particles dismantle and the DNA genome is transferred into the nucleus pore complex (Greber, Suomalainen et al. 1997).

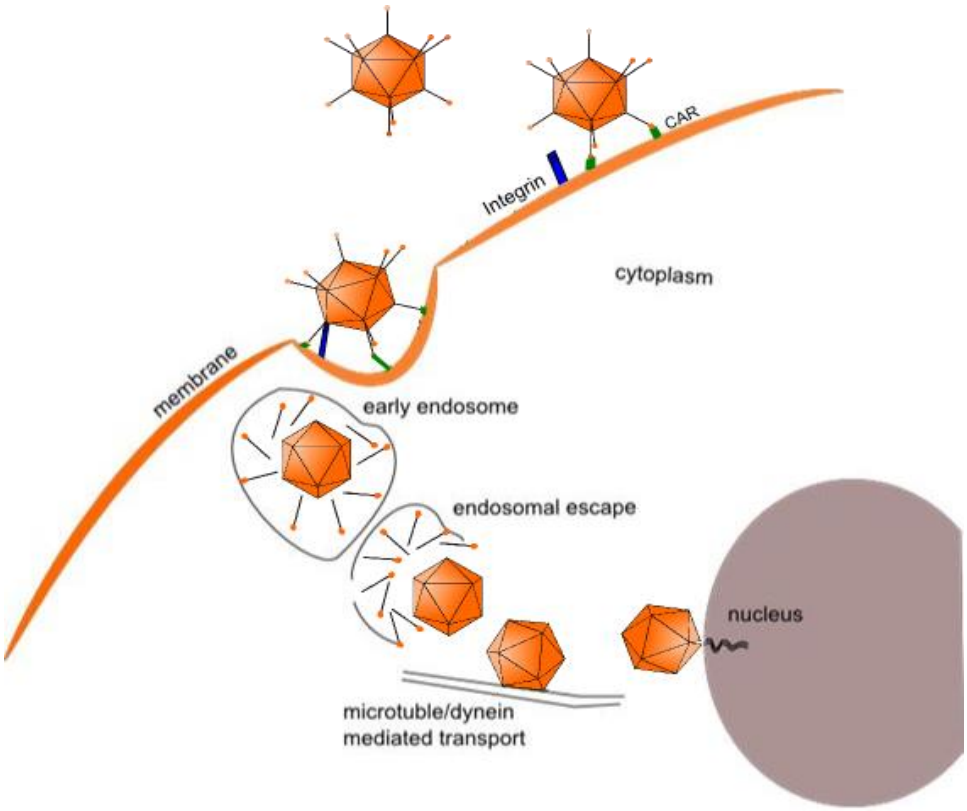

Figure 2.2: Infection pathway of adenovirus. First, the fiber protein of the virus binds to CAR receptor. The Penton base binds to integrin which promotes endocytosis. After cellular internalization, the virus escapes the endosome and binds to microtubule/dynein to be transported to the nucleus. The virus disassembles and releases the viral gene at close proximity to the nuclear pore. The viral gene is then taken up by the nucleus.

2.2. Comparison of Adenovirus with Other Viral Vector

Adenovirus is the most widely used gene delivery vector. Its ability to carry a large gene and infect both dividing and nondividing cells, makes adenovirus a promising gene delivery vector (Benihoud, Yeh et al. 1999, Mizuguchi and Hayakawa 2004). In addition,

the adenovirus genome does not integrate to the host chromosome but exists as an episome. Thus, there is no safety concern in regards to insertional mutagenesis. This property also allows the virus to maintain high levels of gene expression for a short period of time. By deleting E1 and E3 genes, the viral replication and susceptibility of transduced cells to an immune response can been reduced (McConnell and Imperiale 2004). The fiber and capsid protein and some transcription units of the virus, however, elicit immune responses (Kafri, Morgan et al. 1998, Schagen, Ossevoort et al. 2004).

Retroviruses are the most widely used gene delivery agents next to adenovirus. Unlike adenovirus, the retrovirus genome integrates the host cell genome, and the vector produces a long term gene expression (Kay, Glorioso et al. 2001). The low level of immune response towards the virus and infected cells make retrovirus a good gene delivery vector (Cornetta, Morgan et al. 1991). This vector, however, carries relatively small genetic material, and can only transform dividing cells. While the viral genome integration to the host genome allows retrovirus to produce a long term gene expression, it may also cause insertional mutagenesis (Roe, Reynolds et al. 1993).

A retroviral vector, lentivirus, (a subcategory of the retrovirus family) possesses some advantages over other retroviruses. Lentivirus can infect both dividing and nondividing cells and can offer short and long-term transgene expression (Vigna and Naldini 2000). The vector can carry larger insertions compared to AAV. Compared to adenovirus, however, lentivirus has a relatively small gene carrying capacity (Kay, Glorioso et al. 2001).

Adeno-associated virus (AAV) is a nonpathogenic virus with low immunogenicity (Monahan and Samulski 2000). This virus, however, normally requires a helper virus, such as adenovirus, to mediate a productive infection. Similar to adenovirus, AAV can transform both dividing and nondividing cells (Miao, Snyder et al. 1998). The vector can also transduce cells through episomal gene expression and by random chromosomal integration. Compared to adenovirus and retrovirus, however, AAV has limitations on the size of the gene it can transport.

Herpes simplex virus (HSV) is different from the previous vectors because of its specificity to carry large gene fragments to a neuronal cell (Ribotta 2001). The ability of this vector to stay latent until the manifestation of suitable conditions, allows the vector to infect neural cells (Krisky, Marconi et al. 1998). Without these conditions, the vector has low transduction.

2.3. Adenovirus as a Gene Delivery Vector

Adenovirus-based vectors are considered relatively safe and are currently used in approximately 22.5% of the ongoing gene therapy clinical trials (Benihoud, Yeh et al. 1999, Edelstein 2014). However, the immunogenicity of the fiber and capsid protein of the virus may cause an inflammatory response (Kafri, Morgan et al. 1998), and preexisting immunity may initiate clearance of the virus in patients (Chirmule, Propert et al. 1999). Immune inactivation and clearance of the adenovirus is the result of neutralizing antibodies against epitopes on the surface of the virus (e.g. hexon and fiber proteins) (Yang, Li et al. 1995, Sumida, Truitt et al. 2005). A majority of people have circulating antibodies against common adenovirus serotypes (Schmitz, Wigand et al. 1983), and those that do not possess

neutralizing antibodies will develop resistance against reinfection if exposed to an adenovirus vector at a high concentration (Smith, White et al. 1996). From a general health perspective, it is advantageous to have long-term immunity against adenovirus infection, but from a gene delivery standpoint, the innate immune response to the virus limits the efficacy of an adenovirus vector.

Table 2.1: Comparison of viral vectors

Viral vector	Gene delivery efficiency	Insert size	Major advantages	Major disadvantages
Adenovirus	High	< 30 Kb	transform both dividing and nondividing cells; nonpathogenic; nononcogenic	immunogenicity; promiscuous tropism; transient gene expression
Retrovirus	High	< 8 Kb	long term gene expression on dividing cells; relatively low immunogenicity	low in vivo delivery; concern of insertional mutagenesis
Adeno-associated virus	High	< 4.5 Kb	long term gene expression; low immunogenicity	can only carry small size genetic material; concern of insertional mutagenesis
Lentivirus	High	< 8-9 Kb	transform both dividing and nondividing cells	pathogenic
Herpes simplex virus	Low	< 20 Kb	remain latent; carry large genetic material fragment	transient gene expression; cytotoxic; pathogenic; specific to neuronal cell

Tropism of adenovirus is dependent on the presence of a CAR receptor. The ubiquitous presence of CAR on a wide range of tissues hinders the use of adenovirus for targeted

delivery (Mizuguchi and Hayakawa 2004, Campos and Barry 2007). Furthermore, nonspecific binding between motifs present on the virus and proteins on the cell membrane can induce promiscuous tropism. For example, RGD motif on the penton base of the virus binds to integrin receptors, while KKTK motifs on the fiber protein bind to heparan sulfate proteoglycans, leading to a wide tropism (Wickham, Mathias et al. 1993, Smith, Idamakanti et al. 2003) (Fig. 2.3).

Adenovirus fiber protein binding to erythrocytes that express CAR, cause hemagglutination that traps virus in circulation (Henaff, Seiradake et al. 2009). The fiber and capsid protein of the virus also binds to platelets and complements that promote the opsonization of the adenovirus by Kupffer cells (Stone, Liu et al. 2007, Xu, Tian et al. 2008). The complements also trigger inactivation of the virus by Immunoglobulin M. Studies have shown that RGD motif binds to coagulation factor X and leads uptake of the virus by hepatocyte transduction heparan sulfate proteoglycan (Waddington, Mcvey et al. 2008). Adenovirus fiber and capsid also interact with cells of the immune system, such as T-cells, that induce cellular immune response and a cascade of signaling that ultimately destroys the virus (Onion, Crompton et al. 2007).

The death of Jesse Gelsinger, a patient suffering from Ornithine Transcarbamylase (OTC) deficiency, after gene therapy treatment is a sad reminder of how adverse the side effects of these viral drawbacks can be. Studies have since found that the high dose adenovirus administered to the patient invaded unintended targets which subsequently triggered an innate immune response (Marshall 1999). This incident caused doubt about the safety and effectiveness of adenovirus as a gene delivery vector, and gene therapy as a whole. Recently, adenovirus-based vectors have been extensively used in gene therapy. In fact,

two adenoviral gene therapy products are now approved for commercial use in China. In the US, adenoviral vectors are successfully being applied in cancer gene therapy clinical trials. The following section discusses some of the methods to produce a safe and efficient adenoviral gene delivery vector.

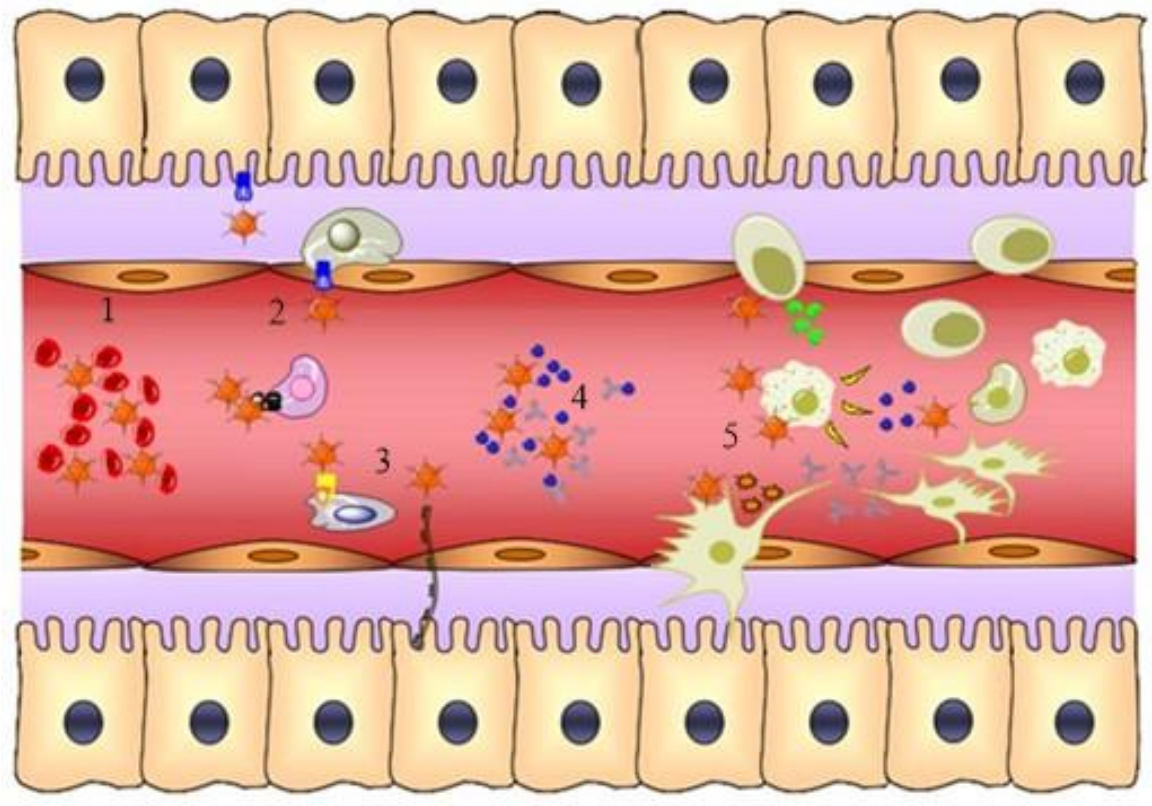

Figure 2.3: Interaction of adenovirus with blood components and cells. Adenovirus fiber capsid protein binds to (1) erythrocytes, (2) CAR, Fc, and scavenger receptor A, (3) coagulation factor X, (4) platelets and complements, and (5) cells of immune system such as T cells.

2.4. Genetic Modification of Adenovirus

The tropism and immunogenicity of adenovirus can be addressed by modifying the virus at the genetic level. First generation adenovirus with its E1 and E3 gene deleted, making the virus replication deficient, is a common type of adenovirus vector used in a wide range

of applications. Further genetic modification of the virus by deleting E2 and E4 regions produces second generation adenovirus with increased packaging capacity. The crucial role played by fiber protein in the promiscuous tropism of the virus prompts the need for further genetic modification. Manipulation of the fiber of adenovirus, such as complete removal of the knob domain (Magnusson, Hong et al. 2001) and genetic replacement of fiber with other serotypes (Krasnykh, Mikheeva et al. 1996, Denby, Work et al. 2004, Tsuruta, Pereboeva et al. 2008) has been shown to ablate or alter the viral tropism. Our group produced a library of recombinant adenoviruses with different truncated fiber proteins to address the promiscuous tropism of the virus. Out of these recombinant adenoviruses, fiberless adenovirus showed the highest stability and the best potential to be used as a gene delivery vector.

2.5. PEGylation of Adenovirus

One of the most common approaches used to reduce the susceptibility of the virus to immune inactivation has been to coat the surface of the virus with neutrally charged polyethylene glycol (PEG). PEG, a safe and biocompatible polymer, is commonly used to prolong the circulatory lifetime of proteins, drugs, and nanoparticles (Kochendoerfer 2003, Otsuka, Nagasaki et al. 2003). Coating of particles with PEG, a technique known as PEGylation, has been shown to improve the tendency of the particles to evade immune recognition and reduce their accumulation in the liver and spleen (Aliabadi, Brocks et al. 2005). In a similar manner, PEGylation of adenovirus has been shown to reduce recognition by neutralizing antibodies, reducing innate immune responses, and altering virus tropism (Chillon, Lee et al. 1998, O'Riordan, Lachapelle et al. 1999, Romanczuk, Galer et al. 1999, Fisher, Stallwood et al. 2001, Lanciotti, Song et al. 2003, De Geest,

Snoeys et al. 2005, Mok, Palmer et al. 2005, Wortmann, Vohringer et al. 2008). In one study, as few as 5,000 PEG molecules per adenovirus were shown to shield the virus from neutralizing antibodies and reduce the innate immune response (De Geest, Snoeys et al. 2005). This degree of PEGylation, however, had little effect on the interaction of the fiber protein of adenovirus toward cellular CAR. Another study demonstrated that in order to reduce the affinity of the virus to CAR-expressing tissues, the virus had to be coated with more than 15,000 PEG/Ad (Mok, Palmer et al. 2005). This level of PEGylation, however, reduced the capability of the virus to effectively transform cells. Our approach is to restore the gene delivery efficiency of adenovirus lost by PEGylation, while keeping the beneficial properties of the PEG coating. Previously, our lab has shown that, when used in combination with PEGylation, materials such as cationic polymer can augment transduction and address issues of reduced gene delivery efficiency (Singarapu, Pal et al. 2013).

2.6. Modification of Adenovirus using Cell-Penetrating Peptides

Adenovirus fiber proteins play a role in the promiscuous tropism and susceptibility of the virus to neutralizing antibodies. Therefore, it would be desirable to replace the fiber proteins with an alternative material that would perform the functions of the proteins (i.e. primarily target cell attachment and cellular entry) without the associated drawbacks. This study proposes that Cell-Penetrating Peptides (CPP) can augment the transduction efficiency of PEGylated adenovirus and can also be used to replace the function of the fiber protein for adenovirus particles. CPPs are an exciting development in the field of drug delivery. CPPs have been used to deliver peptides (Hawiger 1999), large proteins (Schwarze, Ho et al. 1999), plasmid DNA (Singh, Bisland et al. 1999), and most

importantly for our application, nanoparticles (Lewin, Carlesso et al. 2000, Torchilin, Rammohan et al. 2001, Tseng, Liu et al. 2002). CPPs are short peptides, typically less than 30 amino acids, which are capable of inducing the movement of cargo across the cell membrane of mammalian cells (Zorko and Langel 2005). These peptides were initially identified as part of larger proteins that were found to efficiently enter cultured cells (e.g., HIV-1 trans-activating activator of transcription protein). They are categorized as either polycationic peptides, with highly cationic peptides enriched with arginine and lysine, or amphipathic with basic and hydrophobic amino acids clusters, which can interact with the lipophilic region of the cell membrane (Deshayes, Decaffmeyer et al. 2008). Studies have shown that Tat and Penetratin (Pen) CPPs could be used to increase the uptake of liposomes, and improvement in translocation was observed for liposomes with as few as five CPPs per particle (Torchilin, Rammohan et al. 2001, Tseng, Liu et al. 2002). Our previous work, among others, has shown the transduction efficiency of adenovirus was improved by electrostatically binding CPPs to the virus (Gratton, Yu et al. 2003, Lehmusvaara, Rautsi et al. 2006, Nigatu, Vupputuri et al. 2013). The different type of CPPs used to augment the transduction efficiency of adenovirus are discussed below.

2.6.1. Penetratin

Penetratin is a 16-residue peptide derived from *Drosophila melanogaster* Antennapedia homeodomain protein (Derossi, Calvet et al. 1996). Penetratin is an amphipathic peptide composed of positively charged basic amino acids scattered throughout the hydrophobic residues. The cellular uptake of Penetratin is not well understood, but recent studies have suggested that the binding of peptide to cell surface glycosaminoglycans promotes endocytosis (Drin, Cottin et al. 2003, Fischer, Kohler et al. 2004). Penetratin has been

widely used to facilitate cellular delivery of molecules such as siRNA (Lundberg, El-Andaloussi et al. 2007) and oligonucleotides (Astriab-Fisher, Sergueev et al. 2002). We, as well as others, have shown that electrostatically complexing Penetratin with adenovirus can augment the transduction efficiency of the virus (Gratton, Yu et al. 2003, Lehmusvaara, Rautsi et al. 2006, Nigatu, Vupputuri et al. 2013).

2.6.2. Tat

Tat is a 12-residue peptide segmented from an HIV 1 trans-activating protein (48-60) (Vives, Brodin et al. 1997). Tat is mainly composed of nuclear localization sequence and basic amino acids, and has shown the ability to facilitate effective membrane translocation. The initialization pathway of Tat has been debated for years. Recent studies have shown that Tat bound to anionic glycosaminoglycans found on the cell membrane and taken up by macropinocytosis and/or Clathrin-dependent endocytosis (Richard, Melikov et al. 2003, Wadia, Stan et al. 2004, Ziegler and Seelig 2004). Studies have demonstrated that Tat can carry a wide range of macromolecules such as liposomes (Torchilin and Levchenko 2003), oligonucleotides (Astriab-Fisher, Sergueev et al. 2002), and plasmid DNA (Rudolph, Plank et al. 2003) into a variety of cells. Studies, including ours, have also shown that Tat efficiently translocates adenovirus into a wide range of cells (Gratton, Yu et al. 2003, Kida, Eto et al. 2008, Youn, Park et al. 2008, Eto, Yoshioka et al. 2009, Nigatu, Vupputuri et al. 2013).

2.6.3. Polyarginines

Studies have shown that compared to other homopeptides, polyarginine has demonstrated greater cellular uptake (Mitchell, Kim et al. 2000). As few as 8 residue polyarginines produced superior internalization into cells (Futaki, Suzuki et al. 2001). Polyarginine binds

to the cell surface heparan sulphate to promote endocytosis of cargo (Fuchs and Raines 2004). Studies have also reported that a macropinocytosis pathway is a possible route for uptake of polyarginine (Nakase, Niwa et al. 2004). Polyarginines have successfully carried molecules such as siRNA (Kim, Christensen et al. 2006), and plasmid (Kish, Tsume et al. 2007). Similarly, polyarginine has been used to improve the translocation ability of adenovirus (Eto, Yoshioka et al. 2009, Nigatu, Vupputuri et al. 2013).

2.6.4. Pep1

Pep1 is an amphipathic peptide derived from the simian virus 40 T antigens (Morris, Depollier et al. 2001) The Pep1 residue is composed of a hydrophilic lysine rich domain nuclear localization sequence, N-terminal hydrophobic motif, and a linker/spacer. Pep1 translocates into cells by binding to phospholipids on the cell membrane, which may initiate conformational changes for direct translocation (Morris, Depollier et al. 2001). Studies have proven that Pep1 promotes uptake of proteins such as protein Kinase (Maron, Folkesson et al. 2005), antibodies (Morris, Depollier et al. 2001) by different cell lines. Pep1, however, showed relatively limited ability to translocate adenovirus(Nigatu, Vupputuri et al. 2013).

2.7. Targeting of Adenovirus using Ligands

A potential enhancement to an adenovirus-based vector would be to target the vector to specific tissues. This improvement could increase the efficiency of gene delivery to the targeted tissue and reduce nonspecific transduction of untargeted tissues. In the past couple of decades, strategies utilizing unique or over-expressed receptors on the surface of cells to produce targeted vectors have been widely studied. Studies have shown that adenovirus-based vectors can be modified to target specific tissues with the help of ligands that bind

to particular receptors present on the cell membrane (Campos and Barry 2007, Waehler, Russell et al. 2007). The small RGD motif and antibody fragments have been genetically inserted into the capsid or fiber proteins of the adenovirus to facilitate targeted gene delivery (Vigne, Mahfouz et al. 1999, Vellinga, de Vrij et al. 2007). Chemical conjugation of targeting ligands to the capsid of adenovirus, such as covalent coupling of FGF2 via PEG-derived polymers has allowed for adenovirus to be redirected to target FGF2-expressing cells (Lanciotti, Song et al. 2003).

Much effort has been put into targeting immune cells, because these cells play an important role in the progression of an array of diseases. Macrophages are one type of cell widely desired to target in immune and gene therapy (Burke, Sumner et al. 2002). Mannose is one of the most used ligands which binds to specific receptors found in human dendritic and macrophage cells. Polyethylenimine (PEI) and poly-L-lysine (PLL) polyplexes conjugated to mannose showed targeted delivery of DNA to macrophage cells (Diebold, Kursa et al. 1999). Mannose-based targeting of an adenovirus vector to dendritic cells has also proved to be promising (Diebold, Lehrmann et al. 1999). This study aims to incorporate mannose in the formulation of the vector and target macrophage cells.

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CHAPTER III

EFFECT OF CELL-PENETRATING PEPTIDE ON TRANSDUCTION EFFICIENCY OF PEGYLATED ADENOVIRUS

3.1. Introduction

Gene therapy is a promising approach to treating a vast array of diseases. Application of gene therapy, however, has been limited due to the difficulty of developing safe and effective gene delivery vectors. Adenovirus vectors (Ad) are one of the most widely used gene delivery vectors in clinical trials (Ginn, Alexander et al. 2013). High transduction efficiency and the ability to transfer genes into both dividing and non-dividing cells make Ad an attractive choice (Benihoud, Yeh et al. 1999). Most people, however, possess neutralizing antibodies that inactivate Ad (Wohlfart 1988). In addition, administering a high dose of the virus can trigger an inflammatory response (Worgall, Wolff et al. 1997, Kafri, Morgan et al. 1998). Further, Ad infection relies on interaction of the virus with the coxsackie virus and adenovirus receptor (CAR), which limits the ability of the virus to deliver genes into CAR-negative (CAR-) cells such cancer cells, endothelial cells, epithelial cells and smooth muscle cells (Bergelson, Cunningham et al. 1997) As gene therapy advances beyond the initial stages, it is critical to address the drawbacks associated with Ad. Coating the virus with polyethylene glycol (PEG), a technique known as

PEGylation, has been widely applied to improve some of the undesirable properties. Studies have shown that PEGylation Studies have shown that PEGylation of Ad decreases recognition by neutralizing antibodies and reduces the innate immune response to the virus (O'Riordan, Lachapelle et al. 1999, Fisher, Stallwood et al. 2001, Mok, Palmer et al. 2005, Wortmann, Vohringer et al. 2008).Unfortunately, PEGylation also compromises the ability of the virus to transduce cells (Mok, Palmer et al. 2005).

A method for gaining the benefits of PEGylation while retaining infectivity would be beneficial. Further, broadening transduction in a CAR-independent manner would clearly enable use of Ad for a much wider range of diseases. These goals may be achievable through the use of cell-penetrating peptides (CPPs). CPPs are peptides with 7 to 30 amino acid residues and able to transport cargo across the cell membrane into the cytoplasm. CPPs have low cytotoxicity and have the ability to overcome extracellular and intracellular barriers that prevent the transportation of foreign materials into cells (Mae and Langel 2006). CPPs have been shown to transport biomolecules including plasmid DNA, oligonucleotides, siRNA, and proteins with no obvious limitation in the type of cargo (Mae and Langel 2006, Moschos, Williams et al. 2007, El-Sayed, Futaki et al. 2009). In fact, Ad has also been delivered into cells using CPPs such as Tat (Yoshioka, Asavatanabodee et al. 2008, Nigatu, Vupputuri et al. 2013), Penetratin (Pen) (Gratton, Yu et al. 2003, Lehmusvaara, Rautsi et al. 2006, Nigatu, Vupputuri et al. 2013), polyarginine (pArg) (Kim, Kim et al. 2010, Nigatu, Vupputuri et al. 2013) and Pep1 (Kowolik, Yam et al. 2003, Nigatu, Vupputuri et al. 2013).

In this study, we developed a modified Ad vector by conjugating CPP-PEG to virus particles. Vectors were formed by PEGylating Ad and then chemically linking CPPs to the

distal ends of the PEG to form CPP-PEG-Ad particles. Four well characterized CPPs (Tat, Pen, pArg and Pep-1) where selected to identify the best working CPP-PEG-Ad particle. Further, we evaluated variables that affect transduction efficiency such as PEG molecular weight, the degree of PEGylation (DOP), and the ratio of CPP to PEGylated virus. The effect of these variables on the physicochemical properties was also determined.

3.2. Materials and Methods

3.2.1. Cell lines

Human embryonic kidney cells (HEK293) and mouse fibroblast cells (NIH/3T3) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The HEK293 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS) (Mediatech, Inc., Manassas, VA). The NIH/3T3 cell line was cultured in DMEM, with 10% calf serum (CS) (Mediatech, Inc., Manassas, VA). Both cell lines were incubated in a humidified atmosphere at 37 ^{o}C, 5% CO_2.

3.2.2. Adenovirus

Ad with an E1/E3 deletion and expressing the Lac*Z* reporter gene under the control of cytomegalovirus promoter was purchased from Capital Biosciences (Rockville, MD) and amplified using HEK293 cells. Upon showing cytopathic effect, cells were collected and subjected to three freeze/thaw cycles to lyse the cells. The virus was purified from the cell lysate using a Vivapure Adenopack (Sartorius Stedim, Arvada, Colorado). The titer of infective viral particles, in terms of colony forming units (cfu), was determined by infecting HEK293 cells with serial dilutions of the virus and staining for expression of β-

galactosidase. The blue stained colonies were counted using a light microscope with magnification of 40X.

3.2.3. Synthesis of Cell-Penetrating Peptides

Tat (YGRKKRRQRRRC), Pen (RQIKIWFQNRRMKWKKC), pArg (RRRRRRRRRC) and Pep-1 (KETWWETWWTEWSQPKKKRKVC) were synthesized and purified by EZBiolab (Westfield, IN) with an added cysteine residue at the C-terminus of the original peptides. This modification facilitated conjugation of the CPPs to the distal end of a PEG linker without affecting the activity of the CPP.

3.2.4. Synthesis of Polyethylene Glycol

Heterobifunctional PEG (MAL-PEG-NHS) was synthesized and purified by Creative PEGWorks (Winston Salem, NC). One terminal end of the PEG polymer had a thiol-reactive maleimide (MAL) group, and the other terminal end had an amine-reactive N-hydroxyl succinimidyl ester (NHS) group. NHS and MAL enable conjugation of PEG to Ad and the CPPs, respectively. To explore the effect of PEG molecular weight on particle formation and transduction efficiency, MAL-PEG-NHS was purchased with PEG molecular weights of 2, 3.4, 5 and 10 kDa.

3.2.5. Formation of the CPP-PEG-Ad Particles

Formation of a nanoparticle between Ad, PEG and CPP was carried out in two steps (Fig. 3.1). In the first step, PEG linkers were chemically conjugated to Ad particles to form PEGylated Ad (PEG-Ad) through a reaction of the NHS group with the lysine ε-amino groups located on the hexon and penton base proteins of the virus. The PEG was added to

the virus dropwise while gently vortexing. The amount of PEG added ranged from 0 to 9 μg/1 × 10^6 Ad, depending on the desired DOP. The reaction was performed in 50 mM HEPES buffers (pH 8.0) at room temperature for 45 minutes while pulse vortexing every 7-10 minutes. In the second step, CPPs in 20 mM HEPES buffers (pH 7.0) were added to PEGylated Ad and incubated at room temperature for another 45 minutes to form CPP-PEG-Ad particles. The CPPs were conjugated to the MAL functional group of the PEG-Ad particles through a reaction between the thiol-reactive MAL group and the C-terminal cysteine of the CPPs. The amount of CPPs added ranged from 0 to 50 μg/1 × 10^6 PEGylated Ad to produce particles with a range of CPP valencies. In addition to CPP amount and DOP, different types of CPPs and molecular weights of PEG were used to produce CPP-PEG-Ad particles.

3.2.6. Characterization of Conjugation

The amount of PEG conjugated to Ad was estimated by using a fluorescamine assay. Fluorescence produced from the reaction of fluorescamine with free lysine on the virus capsid was used to estimate the DOP. Briefly, 50 μl of fluorescamine (Sigma, St. Louis, MO), at a concentration of 0.6 mg/ml in acetone, were added to in serial dilutions of modified and unmodified virus samples. The reactions were carried out at room temperature for 15 minutes. Fluorescence measurements were then performed using a PTI fluorometer (Photon Technologies International, Edison, NJ), with an excitation wavelength of 390 nm and an emission wavelength of 475 nm. Fluorescence measurements were plotted against virus concentration, and the amount of PEG conjugated was determined by comparing the slopes of the modified to the unmodified virus samples (Stocks, Jones et al. 1986).

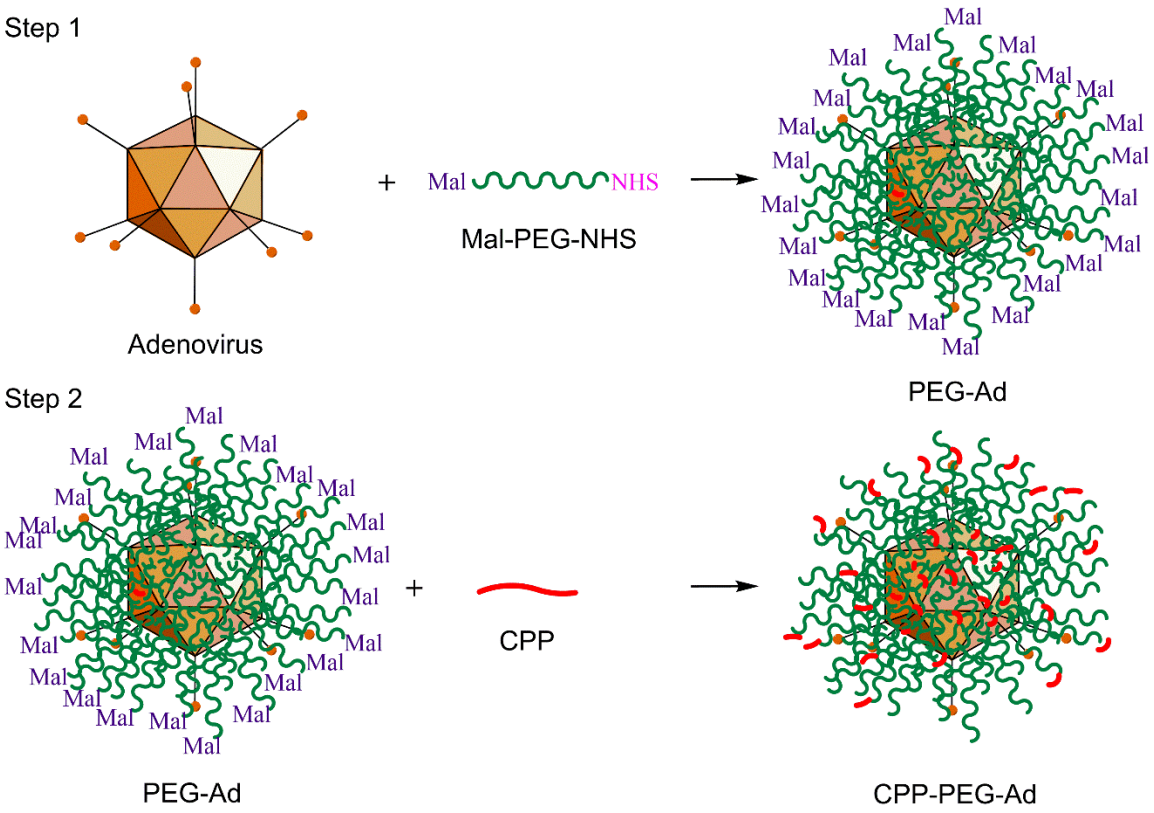


Figure 3.1: Approach for producing CPP-PEG-Ad particles. In Step 1, the N-hydroxyl succinimidyl ester (NHS) chemical group on the heterobifunctional MAL-PEG-NHS reacts with lysine residues in the fiber and capsid protein of the native adenovirus particle to produce PEGylated virus. In Step 2, the thiol-reactive maleimide (MAL) reacts with the cysteine sulfhydryl group on the CPP to produce CPP-PEG-Ad.

An Ellman's assay was used to quantify the amount of free thiol, corresponding to unreacted cysteine residues of CPPs that were not conjugated to the PEG-Ad particles (Ellman 1959, Riddles, Blakeley et al. 1983). The Ellman's reagent, 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB), produces a yellow product, 2-nitro-5-thiobenzoic acid (TNB), when reacted with free sulfhydryl groups, and the product can be quantified by measuring absorbance at 410 nm. The amount of free CPP in the sample was estimated by comparing the sample absorbance to a standard curve composed of known concentrations of a sulfhydryl-containing compound cysteine-HCL.

3.2.7. Particle Size and Zeta-Potential Measurements

A Brookhaven 90Plus dynamic light scattering (DLS) instrument (Brookhaven Instrument, Inc., Holtsville, NY) was used to measure the effective hydrodynamic diameter of the modified virus particles. Samples were diluted in DMEM (pH 7.4) to a concentration of 1×10^7 cfu/ml and the size of the particles was calculated by taking the average of triplicate measurements with six 30 second measurements per sample. The zeta-potential of the particles was measured using a Brookhaven 90Plus ZetaPALS (Brookhaven Instrument, Inc., Holtsville, NY). Samples were diluted in phosphate buffered saline (PBS, pH 7.4) to a concentration 1×10^7 cfu/ml. Zeta-potential measurements were performed in triplicate with 10 repeated measurements per sample.

3.2.8. Transduction Efficiency

The transduction efficiency of CPP-PEG-Ad was studied on CAR- NIH/3T3 cells. The cells were seeded 24 hours before infection at cell seeding density of 2.5×10^6 cells per well in a 12 well plate. All transduction studies were done at an MOI of 50 and in the presence of serum. The transduction level was evaluated using a chemiluminescence-based Beta-Glo assay (Promega Inc., Madison, WI) and a Lumat LB9507 luminometer (EG&G, Berthold, Bundoora, Australia) to quantify the amount of β-galactosidase in terms of relative light units (RLU). Reporter gene expression was then normalized to the total cellular protein content, which was determined from a bicinchoninic acid (BCA) protein assay (Pierce Inc., Rockford, IL).

3.2.9. Statistical Analysis

All results are reported as mean values with standard error calculated from measurements of three or more samples. The statistical analysis between means of two groups was performed using One Way Analysis of Variance (ANOVA) with the Holm-Sidak method. An overall significance level of 95% (i.e., $p < 0.05$) was used to determine statistical significant.

3.3. Results

3.3.1. Effect of CPP Type and Amount on Transduction

To understand how the type of CPP affects transduction of CAR- cells four different CPP-PEG-Ad particles were studied: Tat-PEG-Ad, Pen-PEG-Ad, pArg-PEG-Ad and Pep1-PEG-Ad. The impact of CPP amount and PEG molecular weight was also studied. CPP-PEG-Ad particles were formed by varying the conjugation conditions from 0.1 to 6.25 µg CPP/10^6 PEG-Ad, while maintaining a DOP of 50% in this part of the study (Fig. 3.2). Reporter gene expression from the transformed cells showed that transduction efficiency increased as the amount of CPP increased for all CPP-PEG-Ad particles. Total cellular protein levels, used to normalize reporter gene expression, indicated that the CPP concentration used in this study had no significant effect on cell growth. For all PEG molecular weights, Pen-PEG-Ad showed the greatest increase in transduction (~80 fold) over the tested range of CPP amounts followed by Tat, pArg and Pep1. Transduction efficiency increased most significantly over the low concentration range and improved only slightly as the CPP ratio was increased beyond 1.25 µg CPP/10^6 virus. While the transduction efficiency of most of the CPP-PEG-Ad particles did not show a dependency

on PEG molecular weight, Pen-PEG-Ad efficiency decreased noticeably as the PEG molecular weight was increased from 2 to 5 kDa.

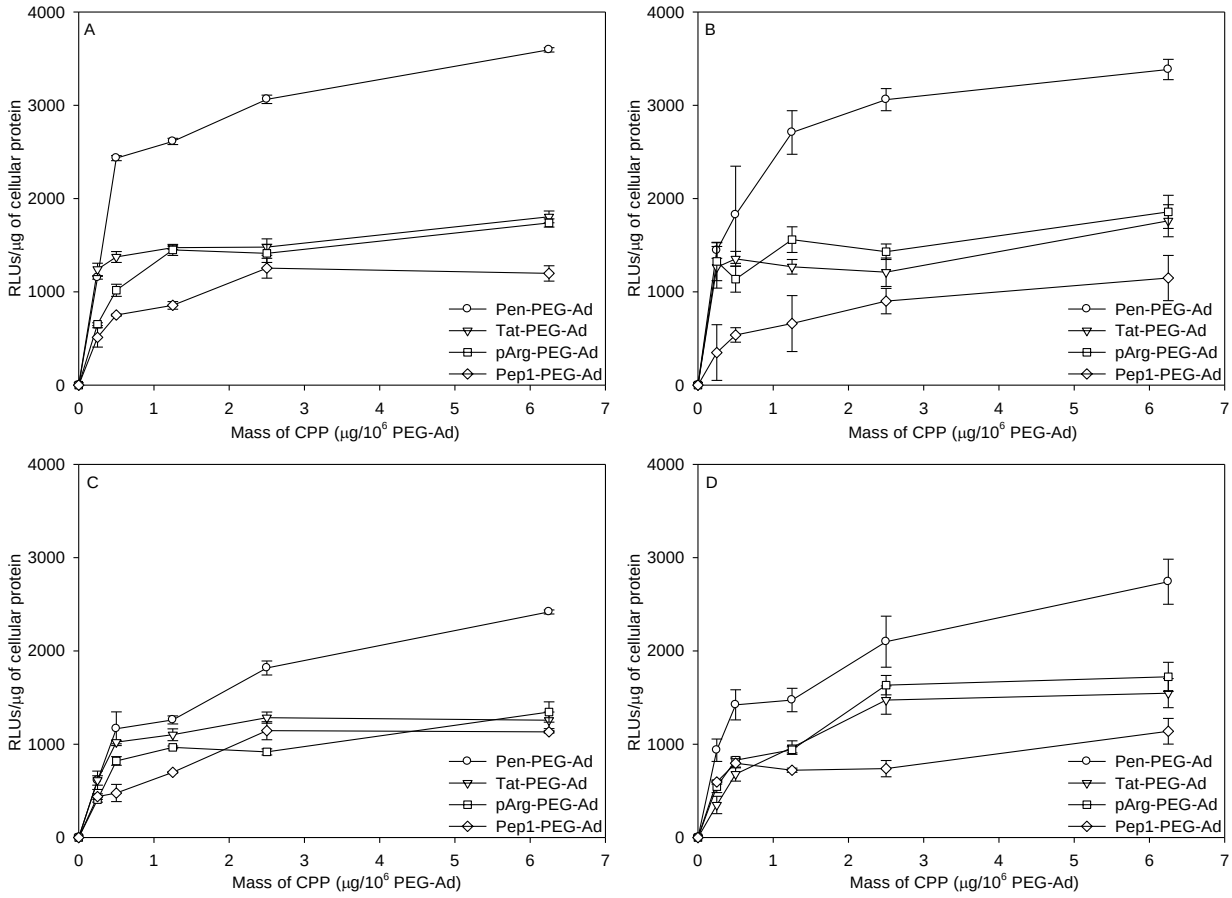


Figure 3.2: Effect of CPP amount on transduction efficiency of CPP-PEG-Ad particles on CAR- NIH/3T3 cells. The CPP-PEG-Ad particles were formed with PEG molecular weights of (a) 2 kDa, (b) 3.4 kDa, (c) 5 kDa and (d) 10 kDa and amounts of CPPs ranging from 0 to 6.25 μg/10^6 PEGylated Ad. All CPP-PEG-Ad particles where modified to 50% DOP. The reported gene expressed from the cells was quantified using a chemiluminescence enzymatic activity assay and was normalize to total cellular protein. The data points represent the mean ± standard deviation (n=3).

The ratio of CPP per PEGylated virus was increased up to 50 μg CPP/10^6 PEG-Ad to test the effect of excess CPP (Fig A3). Under these conditions transduction efficiency of Pen-PEG-Ad did not change. The rest of CPP-PEG-Ad particles showed a gradual increase that ultimately equaled that of the Pen-PEG-Ad particles. The one exception to this trend

was Pep1-PEG-Ad composed of 10 kDa PEG, which showed no improvement at CPP to virus ratios above 6.25 μg/10^6 PEG-Ad.

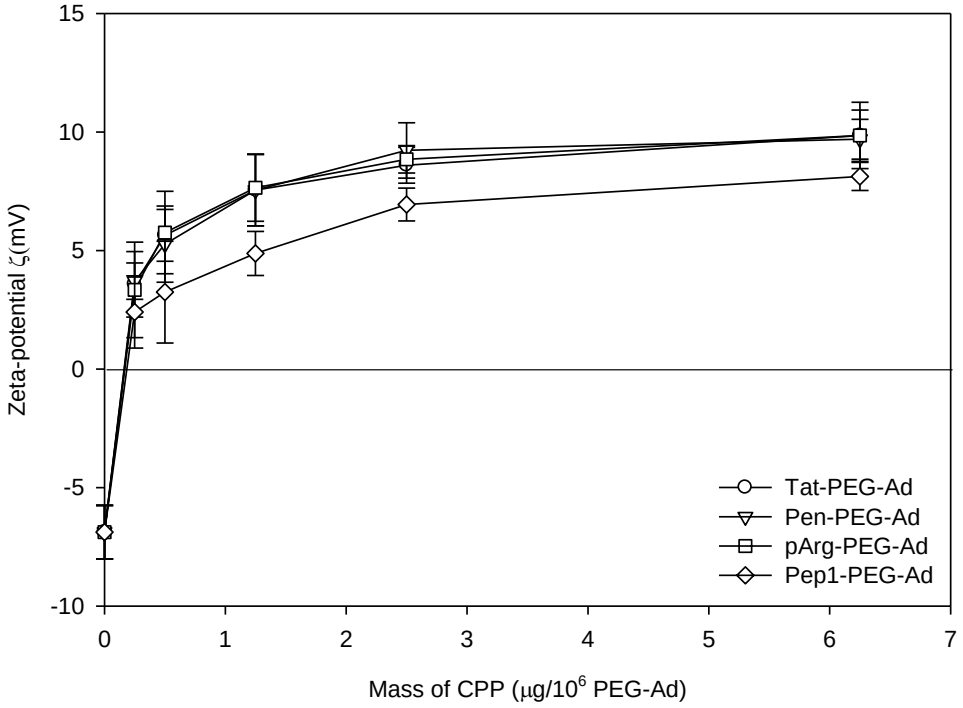


Figure 3.3: Surface charge of CPP-PEG-Ad particles as a function of CPP amount measured by zeta-potential analyzer. The CPP-PEG-Ad particles were formed using a DOP of 50% and 5 kDa PEG molecular weight with the amount of CPPs varied from 0 to 6.25 μg/10^6 PEGylated Ad. The data points represent the mean ± standard deviation (n=6).

3.3.2. Effect of CPP Type and Amount on Physicochemical Properties

Surface charge and particles size are important characteristics for *in vivo* applications. Physicochemical characterization studies were carried out by varying the CPP type and amount to better understand the effect these variables have on the physicochemical properties. Using 5 kDa PEG and a virus particle with 50% DOP, the amount of CPPs was varied from 0.1 to 6.25 μg/10^6 PEG-Ad to form CPP-PEG-Ad. Zeta-potential of the

particles was measured to understand the relationship between surface charge and transduction efficiency of the CPP-PEG-Ad particles. The zeta-potential of the PEG-Ad was -7 ± 2 mV while CPP-PEG-Ad particles had a maximum charge of 10 ± 2 mV (Fig. 3.3). CPP-PEG-Ad showed a substantial increase in zeta-potential up to a CPP amount of 1.25 μg/10^6 virus and increased only gradually beyond this point. Pep1-PEG-Ad produced the lowest average surface charge compared to the other CPP-PEG-Ad particles.

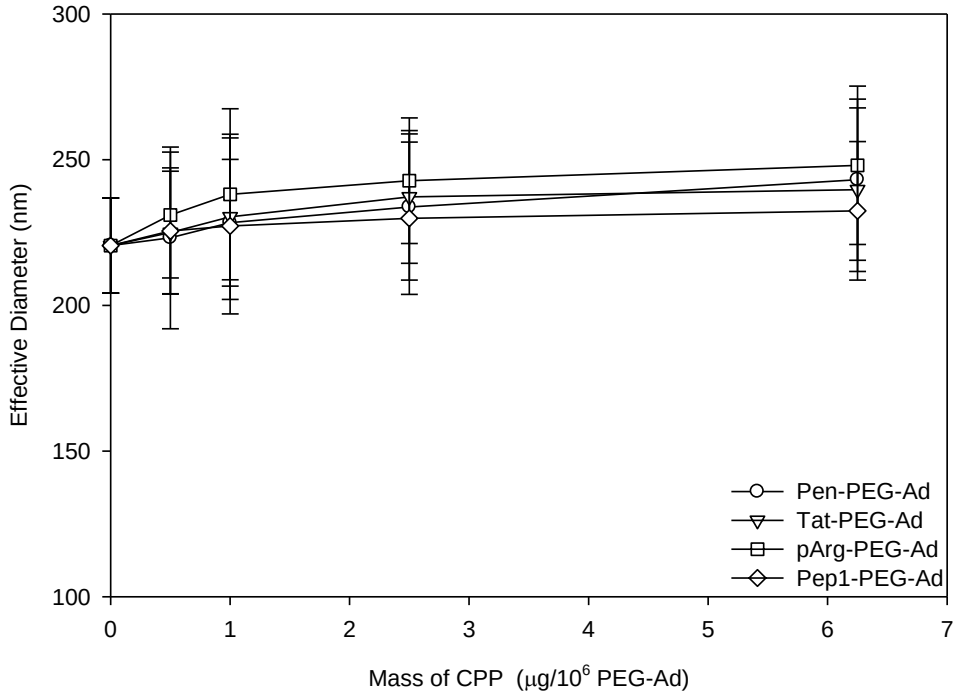


Figure 3.4: Particle size of CPP-PEG-Ad as a function of CPP amount measured using DLS. The CPP-PEG-Ad particles were formed using a DOP of 50% and 5 kDa PEG molecular weight with the amount of CPPs varied from 0 to 6.25 μg/10^6 PEGylated Ad. The data points represent the mean ± standard deviation (n=6).

Particle size affects how well the vector is internalized and can also have adverse effects on the circulatory system if too large. Hydrodynamic diameter of the particles, which was measured using dynamic light scattering, increased upon the addition of CPPs. The

effective diameter of unmodified Ad was found to be 81 nm, which is within the reported range of Ad (Campos and Barry 2007). The size of PEG-Ad was 220 nm and increased to up to 250 nm upon the addition of CPP (Fig 3 4). There was, however, no significant statistical difference between each amount of CPP ($p > 0.05$). Similarly, the particle size was not significantly affected by the type of CPP. The particle size, however, increased up to 380 nm when the particles were incubated for longer than 40 minutes in DMEM containing serum (Fig. 4.8).

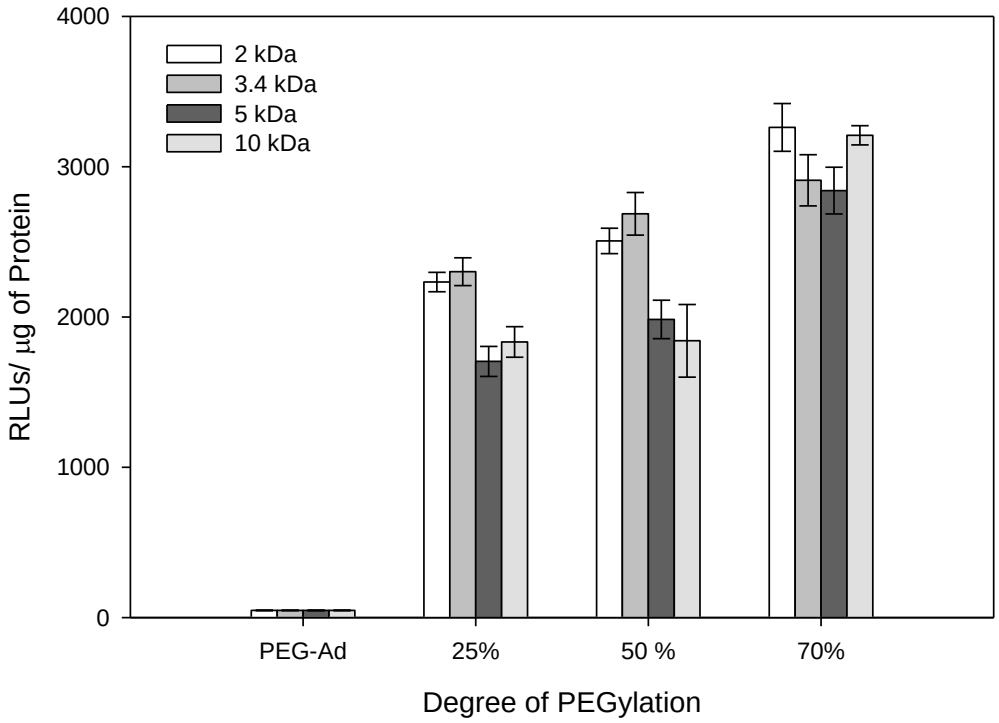


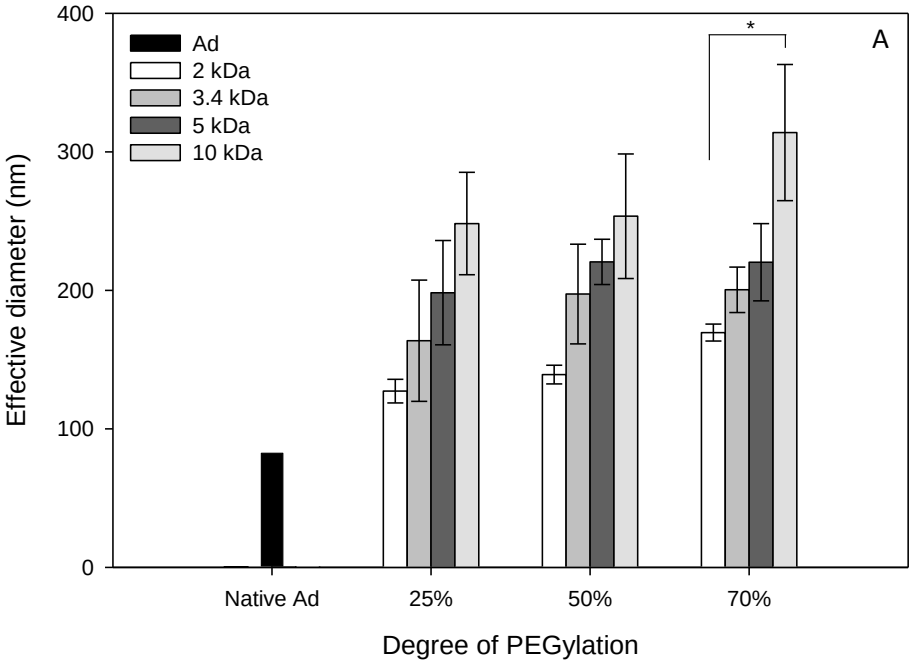
Figure 3.5: Effect of PEG molecular weight and DOP on transduction efficiency of Pen-PEG-Ad particles on CAR- NIH/3T3 cells. The Pen-PEG-Ad particles were formed by PEGylating Ad to 25, 50, or 70% DOP using 2, 3.4, 5, or 10 kDa PEG. The amount of CPP was held constant at 1 µg Pen/10^6 PEGylated Ad. Gene expression was quantified using a chemiluminescence enzymatic activity assay and was normalize to total cellular protein. The data points represent the mean ± standard deviation (n=3).

3.3.3. Effect of DOP and PEG Molecular Weight on Transduction

Pen-PEG-Ad particles were produced by varying the DOP and PEG molecular to evaluate the effect of these PEGylation variables on transduction efficiency. Here, the study used a Pen:virus ratio of 1 μg/10^6 PEG-Ad, which greatly enhanced transduction in the early part of our study (Fig. 3 2). Pen-PEG-Ad particles increased transduction to 3,260 RLUs/mg of cellular protein (Fig. 3.5). At most DOPs the lower molecular weight PEG produced greater transduction than the high molecular weight PEG. Also, the general trend was that for any one molecular weight of PEG, increasing the DOP tended to increase transduction. An exception to this trend was Pen-PEG-Ad particles with 70% PEGylation, where no significant effect on transduction was found for particles produced using different PEG molecular weights.

3.3.4. Effect of DOP and PEG Molecular Weight on Physicochemical Properties of PEG-Ad and Pen-PEG-Ad Particles

Effective hydrodynamic diameter of PEG-Ad and Pen-PEG-Ad particles was measured using DLS to understand the effect of DOP and PEG molecular weight on particle size. The results showed that PEG molecular weight, in most cases, had a significant effect on the size of the particles (Fig. 3.6a). For example, the diameter of 70% PEGylated Ad increased from 169 nm to 313 nm as PEG molecular weight increased from 2 kDa to 10 kDa ($p < 0.05$). In general, the study showed that as PEG molecular weight increased, the DOP had less effect on the size of the particles.



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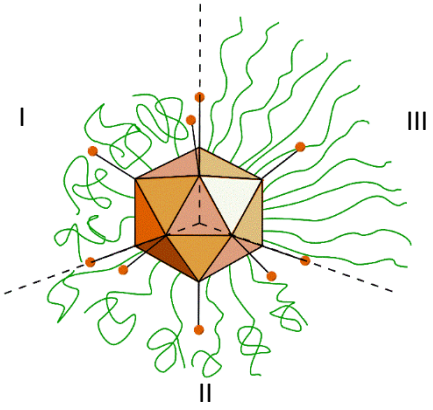

Figure 3.6: A) Particle size of PEG-Ad as a function of PEG molecular weight and DOP measured using DLS. The PEG-Ad particles were formed by PEGylating Ad to 25, 50, or 70% DOP using 2, 3.4, 5, or 10 kDa PEG. The data points represent the mean ± standard deviation (n=6) (*$p < 0.05$). B) The transition of PEG conformations as the DOP increases – mushroom to brush (counter clockwise). Region I shows PEG assuming a "mushroom" conformation when the DOP is relatively low. Region II shows PEG transitioning from "mushroom" to "brush" as the DOP increases. Region III shows PEG assuming the "brush" conformation at a high level of DOP.

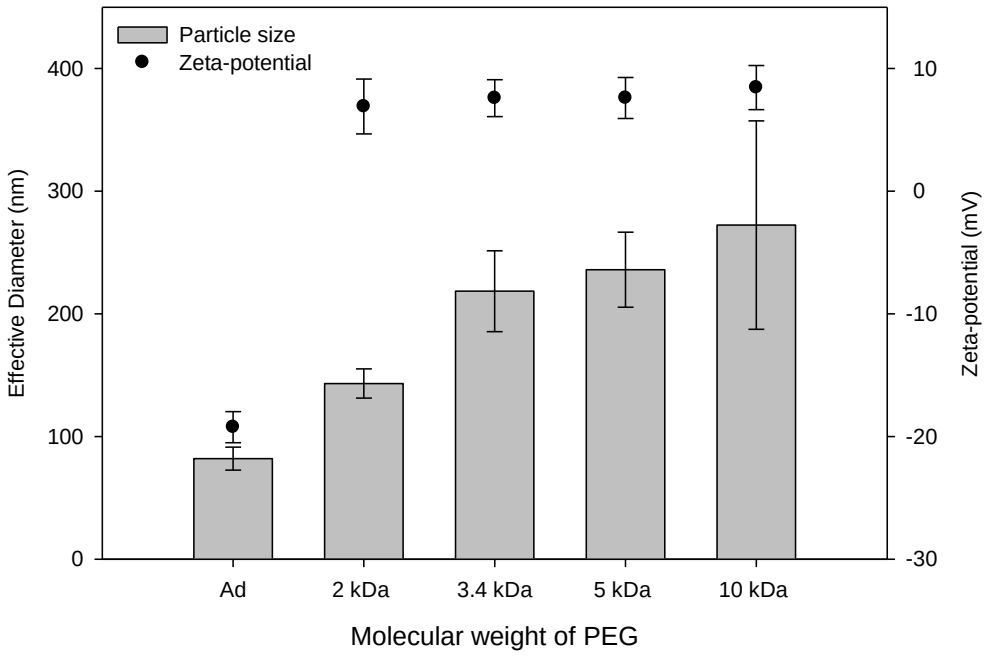

Figure 3.7: Particle size and surface charge of Pen-PEG-Ad as a function PEG molecular weight were measured using DLS and a zeta-potential analyzer. The Pen-PEG-Ad particles were formed by PEGylating Ad to a 70% DOP using PEG molecular weights of 2, 3.4, 5, or 10 kDa and adding 1 µg Pen/10^6 PEGylated Ad. The data points represent the mean ± standard deviation (n=6)

Further characterization of Pen-PEG-Ad with a DOP of 70% and different PEG molecular weights was performed. Both particle size and surface charge were measured over the range of PEG molecular weights. Zeta-potential of Pen-PEG-Ad was measured to be 8 mV and was not significantly affected by the difference in PEG molecular weight. Size of Pen-PEG-Ad particles increased from 181 nm to 330 nm as the PEG molecular weight increase from 2 to 10 kDa. Interestingly, although the size of PEG-Ad increased by about 50% when the PEG molecular weight was increased from 5 to 10 kDa (Fig 3.6a), we only observed a 10% difference in the size of the Pen-PEG-Ad particles composed of 5 kDa PEG compared to particles composed of 10 kDa PEG (Fig 3.7).

3.4. Discussion

Studies have demonstrated that CPPs can be used to successfully augment gene delivery efficiency (Gratton, Yu et al. 2003, Lehmusvaara, Rautsi et al. 2006, Eto, Yoshioka et al. 2009, Nigatu, Vupputuri et al. 2013). Our results extended these findings by testing a small library of CPPs that were conjugated to PEGylated Ad. These CPPs exhibited a strong influence on the transduction efficiency of CPP-PEG-Ad particles on CAR- cells. Transduction efficiency of the particles was also dependent on conjugation parameters such as CPP amount, DOP, and PEG molecular weight.

To understand the effect of these conjugation parameters on the properties of the CPP-PEG-Ad, we first produced particles with different CPP amounts and tested the transduction efficiency and physicochemical properties. Our study showed that the amount of CPP had a significant importance on the ability of the particles to transform cells. For all CPP-PEG-Ad particles, infectivity improved as the CPP to PEG-Ad ratio was increased. The transduction efficiency showed only moderate improvement beyond 1.25 μg CPP/10^6 PEG-Ad (Fig 3.2). This amount of CPP likely corresponded to the effective amount of peptide that could be conjugated to the PEGylated virus at a 50% DOP, and increasing the ratio of CPP to PEG-Ad resulted in CPPs that either electrostatically bound the particles or remained free in solution. Correspondingly, the amount of CPP had a substantial effect on zeta-potential at ratios below 1.25 μg/10^6 virus and only a moderate impact at higher CPP ratios (Fig 3.3). In contrast, the amount of CPP had no significant impact on the size of the CPP-PEG-Ad particles. The relationship between CPP amount and zeta-potential suggests further that at these high CPP valencies, the peptides may have completely bound to PEG attached to the surface of the virus, thereby hindered additional CPPs from binding

to the particle. A similar effect occurred when the amount of CPP added was held constant (1 µg CPP/10^6 PEG-Ad) and the DOP was varied (Fig 3.4). When the number of conjugation sites for the CPPs (i.e., the DOP) increased to 70%, Pen-PEG-Ad particles produced higher transduction efficiency compared to the lower DOP particles. Together these observations indicate that CPPs conjugated to the particles have a much greater impact on the efficiency of the particles than CPPs that either electrostatically bind the particles or remain free in solution.

Noticeably, this study also demonstrated that the type of CPP strongly impacted transduction efficiency of CPP-PEG-Ad particles on CAR- cells. Pen-PEG-Ad produced the greatest increase in transduction. Tat-PEG-Ad and pArg-PEG-Ad, which behaved similarly, both showed significant improvements in transduction but were less effective than Pen-PEG-Ad. Pep1-PEG-Ad also enhanced transduction but was consistently less efficient than the other CPPs under all conditions.

Why one type of CPP performed better than others is not entirely clear. The reasons could range from how the CPPs influence cellular association of the particle to how they influence the internalization pathway. With regard to cellular association, most CPPs are highly positively charged and generally attach to the cell membrane through electrostatics (Honary and Zahir 2013, Honary and Zahir 2013). Studies have shown that the amphipathicity of the peptide also influences cellular association (Dom, Shaw-Jackson et al. 2003, Gros, Deshayes et al. 2006). In terms of the internalization pathway, recent studies indicate that cellular uptake generally occurs through energy-dependent endocytosis (Drin, Cottin et al. 2003, Richard, Melikov et al. 2003, Jiao, Delaroche et al. 2009). While seldom, uptake can also occur through a non-endocytic route and sometimes through a

combination of both an endocytic and non-endocytic route (Deshayes, Morris et al. 2005, Ziegler, Nervi et al. 2005, Zorko and Langel 2005, Duchardt, Fotin-Mleczek et al. 2007, Patel, Zaro et al. 2007).

As mentioned above, electrostatic binding of the CPP-PEG-Ad particles to the cell membrane plays an important role in cellular association of the vector. Because of this importance, the differences in transduction efficiency of CPP-PEG-Ad particles were compared with differences in zeta-potential. The zeta-potential and transduction efficiency of all CPP-PEG-Ad particles showed similar trends, with increasing zeta-potential generally corresponding to increasing transduction efficiency. The results also demonstrated that Pep1-PEG-Ad had the lowest surface charge of the different CPP-PEG-Ad particles and similarly produced the lowest levels of reporter gene expression.

Electrostatic attraction alone, however, is not enough to account for the observed differences since Pen-PEG-Ad, Tat-PEG-Ad and pArg-PEG-Ad all had similar zeta-potentials but had different transduction efficiencies. Instead, these differences may be attributed to how each CPP facilitates interaction between the particles and the cells and the fact that this interaction may not be solely dependent on electrostatics. Most CPPs improve cellular association through electrostatic binding, which occurs with cell-surface glycoproteins such as heparan sulfate (Eto, Yoshioka et al. 2009). Highly positively charged, hydrophilic CPPs, such as Tat and pArg, bind to negatively charged heparan sulfate proteoglycans through electrostatic attachment. Other CPPs, such as Pen, utilize electrostatic binding with cell surface proteoglycans and also hydrophobic interactions with the cell membrane. Lastly, there are some CPPs, like Pep1, that contain distinct hydrophilic and hydrophobic domains that strongly interact with the cell lipid membrane

but have limited electrostatic binding with cell surface proteoglycans (Gros, Deshayes et al. 2006). The combined hydrophobic and electrostatic interactions of Pen with the cell membrane may partly explain why Pen performed better than Tat and pArg, which utilize only electrostatic bindings, and Pep1, which relies predominantly on hydrophobic interactions (Gros, Deshayes et al. 2006).

The internalization routes used by different CPPs may provide yet another explanation for why differences were observed in transduction efficiency. Recent studies have reported that, when conjugated to macromolecules, the internalization of Tat, Pen, and pArg occurs primarily through energy-dependent endocytosis, and the internalization of Pep1 occurs through a nonendocytic mechanism (Magzoub and Graslund 2004, Gros, Deshayes et al. 2006). In the present study, the peptides that used an endocytic route produced much higher levels of transduction than the peptide that used a nonendocytic route. Why particles that utilize the endocytic route performed better is likely tied to how native Ad infects cells. Ad is normally internalized through receptor-mediated endocytosis. The virus is then exposed to a harsh progressive acidification of the endosomal compartment, which triggers conformational changes of capsid proteins and leads to activation of viral protease p23 (Furcinitti, Vanoostrum et al. 1989). This protease cleaves amphipathic capsid proteins with membrane lytic activity and stimulates endosomal escape (Wiethoff, Wodrich et al. 2005). The low pH environment also leads to structural changes that enable motor proteins such as dynein to bind to the virus, once it escapes from the endolysosomal network, and transport the virus particle to the cell nucleus along microtubules (Bremner, Scherer et al. 2009). Further, acidification that takes place in the endosome leads to partial disassembly of the virus and allows viral DNA to be released from the inner wall of the capsid, which

is critical for complete virus disassembly and gene delivery once the virus has reached the cell nucleus. Consequently, a nonendocytic route that avoids exposing the virus to low pH, such as that taken by Pep1-PEG-Ad, prevents disassembly of the viral capsid and binding of the virus to motor proteins that ultimately lead to nuclear localization. Virus particles transported into the cell using peptides that utilize an endocytic route (e.g., Tat, Pen, pArg), continue to be exposed to the low pH environment and undergo the necessary structural changes required for gene delivery to the cell nucleus.

Interestingly, the differences we observed in transduction efficiency between peptides that utilized an endocytic route and the peptide that utilized a nonendocytic route were not observed at high concentrations of the peptide (i.e, 50 μg/10^6 PEG-Ad, Fig A3). In fact, all of the CPP-PEG-Ad particles produced similar levels of transduction at this highest concentration of CPPs. Why Pep1-PEG-Ad particles worked as well as the CPP-PEG-Ad particles that utilize an endocytic route is likely due to differences in how the particles were internalized at high concentrations of CPP. Previous studies have reported that at high concentrations of CPPs, particles tend to be internalized in both an endocytic and nonendocytic manner (Lee, Dubikovskaya et al. 2008, Jiao, Delaroche et al. 2009). In our case, the combination of both endocytic and nonendocytic routes resulted in the Pep1-PEG-Ad particles performing similar to the Pen-PEG-Ad, Tat-PEG-Ad, and pArg-PEG-Ad particles.

Of the four CPPs investigated in our study, Pen peptide produced the most efficient CPP-PEG-Ad particles for gene delivery at reasonable CPP concentrations. These particles were used in the last part of our study to further understand the effects PEG molecular weight and DOP have on the transduction efficiency and the physicochemical properties. Pen

produced with low DOP showed low transduction efficiency compared to moderate and high DOP. In fact, the highest DOP tested in this study (i.e., 70%) produced particles that resulted in the best transduction efficiency. The lower transduction efficiency of the particles with low DOP is attributed to the PEG molecules adopting a "mushroom" conformation, which is observed when PEG chains are not tightly packed on the nanoparticle surface (Levin, Bishnoi et al. 2006, Jokerst, Lobovkina et al. 2011). In this conformation the peptide conjugation sites can be hidden inside the "mushroom" coil, which prevents CPPs from conjugating to the PEGylated Ad. In contrast, a high DOP forces PEG to adopt the "brush" conformation and provides a greater number of more accessible sites for the CPP to conjugate. This conformation also promotes better exposure of the conjugated CPPs on the surface of the particle (Fig 3.6a). The transition of the PEG from the "mushroom" coil to the "brush" was also seen in the particle size study of PEG-Ad, where increasing the DOP resulted in larger particles (Fig 3.6b).

Our evaluation of the physicochemical characteristics showed that Pen-PEG-Ad particles initially had a size appropriate for use *in vivo* (Fig 3.7). When the Pen-PEG-Ad particles were incubated in medium with serum, however, serum proteins adsorbed onto the positively charged particles increasing the hydrodynamic diameter significantly (Fig. 4.8). Clearly, advancements are needed to improve the stability of CPP-PEG-Ad particles in the presence of serum. Additionally, incorporating a targeting ligand will help target Ad to specific cell types and may improve the overall effectiveness of CPP-PEG-Ad particles. This study provides an exciting platform for the further development of hybrid, Ad-based gene delivery vectors.

3.5. Conclusions

In general, the present study demonstrated the potential feasibility of a hybrid, Ad-based gene vector. Chemical modification of PEGylated Ad with CPPs significantly improved the ability to the virus to transform CAR- cells that Ad alone cannot efficiently infect. Pen-PEG-Ad particles produced the highest transduction efficiency compared to Tat-PEG-Ad, pArg-PEG-Ad and Pep1-PEG-Ad particles. The differences in transduction efficiency were attributed to how CPPs facilitated association of the particle with cells and also whether particles were internalized in an endocytic or nonendocytic manner. Further, the results indicated that an endocytic route might be especially important for an Ad-based vector. The study also showed that, at higher degrees of PEGylation, PEG adopted a "brush" conformation that allowed better conjugation of CPPs to the PEGylated virus. The change in conformation and addition of conjugation sites explained why transduction efficiency was generally highest when higher DOPs were used.

CHAPTER IV

CELL-PENETRATING PEPTIDE/POLYETHYLENE GLYCOL CONJUGATES ENHANCE TRANSDUCTION EFFICIENCY AND REDUCE THE IMMUNOGENICITY OF AN ADENOVIRUS VECTOR

4.1. Introduction

Adenovirus (Ad) has generated considerable interest as a potential viral gene delivery vector. Ad has the ability to transduce a wide variety of dividing and non-dividing cells and produces high levels of transgene expression (Benihoud, Yeh et al. 1999, Mizuguchi and Hayakawa 2004, Campos and Barry 2007). The virus, however, has some major drawbacks. The fiber and capsid proteins of the virus are recognized by cells of the immune system and induce an immune response that includes the release of pro-inflammatory cytokines, silencing of gene expression, and clearance of the virus from the body (Kaplan, StGeorge et al. 1996, Kafri, Morgan et al. 1998). Pre-existing immunity also prevents administration of the vector in patients with prior exposure (Kaplan, StGeorge et al. 1996, Christ, Lusky et al. 1997). Another drawback is the broad and promiscuous tropism of the virus. The initial cellular attachment of Ad is primarily dependent on the ubiquitously found coxsackie and adenovirus receptor (CAR), which results in widespread infection of many cell types if administered systemically (Bergelson, Cunningham et al. 1997).

Dependency on this receptor also limits the ability of Ad-based vectors to transform cells that lack CAR, such as advanced tumor cells, peripheral blood cells, and vascular smooth muscle cells. Because of thesenumerous drawbacks, clinical applications of Ad-based vectors have been hindered (Wohlfart 1988, Mastrangeli, Harvey et al. 1996).A number of studies have successfully demonstrated strategies to reduce the susceptibility of the vector to the immune system. One approach has involved encapsulation of the virus within liposomes to hide it from the immune system (Steel, Cavanagh et al. 2007), but the most popular approach has been to use polyethylene glycol (PEGylation) to modify the surface characteristics of the virus (Wonganan and Croyle 2010). Studies have shown that coating the virus with PEG protects the virus from neutralizing antibodies and reduces recognition of viral epitopes by cells of the immune system (O'Riordan, Lachapelle et al. 1999, Fisher, Stallwood et al. 2001, Mok, Palmer et al. 2005)**.**

PEGylation has not only played an important role in addressing the immunogenicity of the virus but has also been used to help address problems with viral tropism. Studies have shown that PEGylation reduces the affinity of the virus for its natural receptor (Mok, Palmer et al. 2005). Oh *et al*., for example, produced a PEGylated Ad with folic acid that showed reduced levels of uptake by lung cells and greater transduction of epithelial cancer cells (Oh, Mok et al. 2006). Genetic manipulation of the capsid and fiber/knob proteins has also shown some success in altering the natural tropism of the virus. Studies have shown that genetically inserting a cell-penetrating peptide (CPP), such as Tat, into the HI loop of the knob portion of the fiber protein enhances transduction of cells that lack CAR (Kurachi, Tashiro et al. 2007). CPPs have also been chemically conjugated to the Ad capsid protein and demonstrated to improve transduction efficiency (Kida, Maeda et al. 2006, Eto,

Yoshioka et al. 2009). Our group, among others, has shown that even simply complexing CPPs to the virus through electrostatic interactions seems to be sufficient for improving the translocation of Ad into a wide range of cell types (Gratton, Yu et al. 2003, Lehmusvaara, Rautsi et al. 2006, Youn, Park et al. 2008, Park, Doh et al. 2010, Nigatu, Vupputuri et al. 2013).

Modification of Ad with CPPs alone, however, does not address the tendency of the virus to elicit an immune response. Instead, a combination of the approaches described above is needed to see the benefits of both reduced immunogenicity and improved transduction of CAR-negative (CAR-) cells. Studies have shown that a reduced immune response can be achieved by tethering RGD peptides to Ad *via* PEG linkers (Ogawara, Rots et al. 2004, Eto, Gao et al. 2005). These studies, however, did not investigate other CPPs or compare the effects of PEG molecular weight on immunogenicity and transduction efficiency. Consequently, the combined effects of these parameters on the immunogenicity and transduction efficiency are not well understood.

To better understand how the type of CPP and PEGylation affects transduction efficiency, we recently developed an Ad-based vector by conjugating CPPs to the distal end of PEGylated Ad (PEG-Ad) (Nigatu, Flynn et al.)**.** The focus of the present study was to better understand how the same parameters (type of CPP, DOP, and PEG molecular weight) affect immunogenicity**.** Instead of exploring the entire range of conditions used in the earlier study**,** the two best performing CPPs (Penetratin (Pen) and Tat), two DOPs (50 and 75%), and two PEG molecular weights (5 and 10 kDa) were used to produce the CPP-PEG-Ad particles. The effect of parameters on **s**usceptibility of the vector to inactivating antibodies as well as its tendency to induce an immune response was evaluated. The benefit

PEGylation imparts on stability of the vector was evaluated by comparing the CPP-PEG-Ad particles to the electrostatically formed CPP/Ad complexes and unmodified Ad. Transduction efficiency of the particles was also studied on both CAR-expressing (CAR+) and CAR-negative (CAR-) cell lines.

4.2. Materials and Methods

4.2.1. Cell Culture

Mouse fibroblast cell line (NIH/3T3), mouse leukaemic monocyte macrophage cell line (RAW264.7), and human embryonic kidney cell line (HEK293) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The HEK293 and RAW264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Inc., Manassas, VA). The NIH/3T3 cells were cultured in DMEM with 10 % calf serum (CS) (Mediatech, Inc., Manassas, VA). Cells were maintained in a humidified atmosphere at 37^{o}C and 5% CO_2.

4.2.2. Adenovirus

Recombinant Ad that has an E1/E3 gene deletion and expresses the *lacZ* reporter gene was acquired from Capital Biosciences (Rockville, MD). The virus was amplified by infecting HEK293 cells, and the infected cells were harvested after showing signs of the cytopathic effect. The cells were then lysed with three freeze/thaw cycles. The resulting virus stock was purified using a Vivapure Adenopack purification kit (Sartorius Stedim, Arvada, CO). The final titer of infective Ad particles was determined in terms of colony forming units

(cfu), by infecting HEK293 cells with serial dilutions of the virus and counting the blue cells after 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) staining.

4.2.3. Synthesis of Cell-Penetrating Peptides and Polyethylene Glycol

Pen (RQIKIWFQNRRMKWKKC) and Tat (YGRKKRRQRRRC) peptides were produced and purified by EZBiolab (Westfield, IN) with a cysteine residue on the C-terminus end of the peptide to facilitate conjugation with a PEG linker. Aliquots of the CPPs were prepared in PBS buffer at a concentration of 2 µg/µl. Heterobifunctional PEG (MAL-PEG-NHS) with molecular weights of 5 and 10 kDa was purchased from Creative PEGWorks (Winston Salem, NC). One terminal end of the heterobifunctional PEG had a thiol-reactive, maleimide (MAL) group, and the other end had an amine-reactive, N-hydroxyl succinimidyl ester (NHS) group. The NHS enabled the conjugation of PEG to Ad while the MAL functional groups allowed the binding of PEG with CPPs.

4.2.4. Formation of CPP/Ad Complexes and CPP-PEG-Ad Particles

CPP/Ad complexes, which were used as a control to understand the effects PEGylation, were formed through non-covalent binding of Ad and CPPs (Fig. 4.1A). The positively charged CPPs were electrostatically attached to the negatively charged Ad. Ad particles were diluted in HEPES buffer (pH 7.4; 20 mM) to a concentration of 1×10^6, and 6.25 µg of CPP (or 12.5 µl of the CPP working solution) was added to the solution. Complexes were formed by incubating the solution at room temperature for 1 hour.

CPP-PEG-Ad particles were produced in a two-step process (Fig. 4.1B). In the first step, PEG was chemically conjugated to Ad through reaction of the PEG-NHS and with the

lysine residues located on the virus, resulting in PEGylated Ad (PEG-Ad). The reaction was carried out by adding 4 to 8 µg of 5 kDa or 10 kDa PEG, dropwise, to 1×10^6 Ad in 100 µl of HEPES buffer (pH 8.0; 50 mM). The amount of PEG conjugated to Ad was varied to produce PEG-Ad with different DOP. The reaction was performed at room temperature for 45 minutes. In the second step, CPPs were attached to the PEGylated virus through reaction of the PEG-MAL with the cysteine residue incorporated into the peptides. The CPP-PEG-Ad conjugates were formed by adding 6.25 µg of CPP (Tat or Pen) to the 1×10^6 PEG-Ad particles. The reaction was performed at room temperature for 45 minutes in 150 µl of HEPES buffers (pH 7.0; 20 mM). A fluorescamine assay and Ellman's assay were used to quantify the conjugation of PEG to Ad and CPP to PEG-Ad, respectively, following published protocols (Nigatu, Vupputuri et al. 2013).

4.2.5. Preparation of Ad Antiserum

Female Gab mice were immunized by subcutaneous injection with 50 µl of Ad vector that contained 1×10^9 infectious virus particles. Four weeks later, the mice were treated with a booster injection with the same amount of virus as the first immunization. Sera were collected from five control mice and five immunized mice 4 weeks after the booster injection and used for antibody neutralization studies. All animal procedures were approved by the Institutional Animal Care and Use Committee of Oklahoma State University.

4.2.6. Transduction Efficiency Studies

The transduction efficiency of Ad, CPP/Ad complexes, and CPP-PEG-Ad was studied on NIH/3T3, and HEK293 cells. Cells were seeded 24 hours prior to infection at 2.5×10^6

cells per well on 12 well plates. Before infection, the cell culture medium was replaced with fresh medium with or without serum. The cells were then infected with the produced viruses using an MOI of 2 for HEK293 cells and an MOI of 100 for NIH/3T3 cells. Four hours later the medium was again replaced with fresh cell culture medium and cells were incubated for 48 hours. *Lac*Z expression was quantified using the chemiluminescence-based Beta-Glo assay (Promega Inc., Madison, WI). The reporter gene expression was measured in terms of relative light units (RLU) with a Lumat LB9507 luminometer (EG&G, Berthold, Bundoora, Australia). The gene expression was then normalized to total cellular protein determined using the bicincroninic acid assay (BCA assay) (Pierce, Rockford, IL).

4.2.7. Cytotoxicity of CPP

The viability of the cells infected with CPP-PEG-Ad was studied using Cell Titer Blue Cell Viability Reagent (Promega Inc., Madison, WI). Briefly, NIH/3T3 cells were seeded in 96-well plates at a seeding density of 2.5×10^4 cells per well and incubated at 37°C for 24 hours. CPP-PEG-Ad particles were prepared with CPP concentrations ranging from 0.1 to 50 µg per 1×10^6 Ad. The seeded cells were incubated with CPP-PEG-Ad at 37°C for 10 hours. Cells were then incubated with the reagent for 4 hours at 37°C and fluorescence measurements performed at an excitation wavelength of 560 nm and an emission wavelength of 590 nm using a SpectraMax Gemini XPS spectrophotometer (Molecular Devices, Sunnyvale, CA).

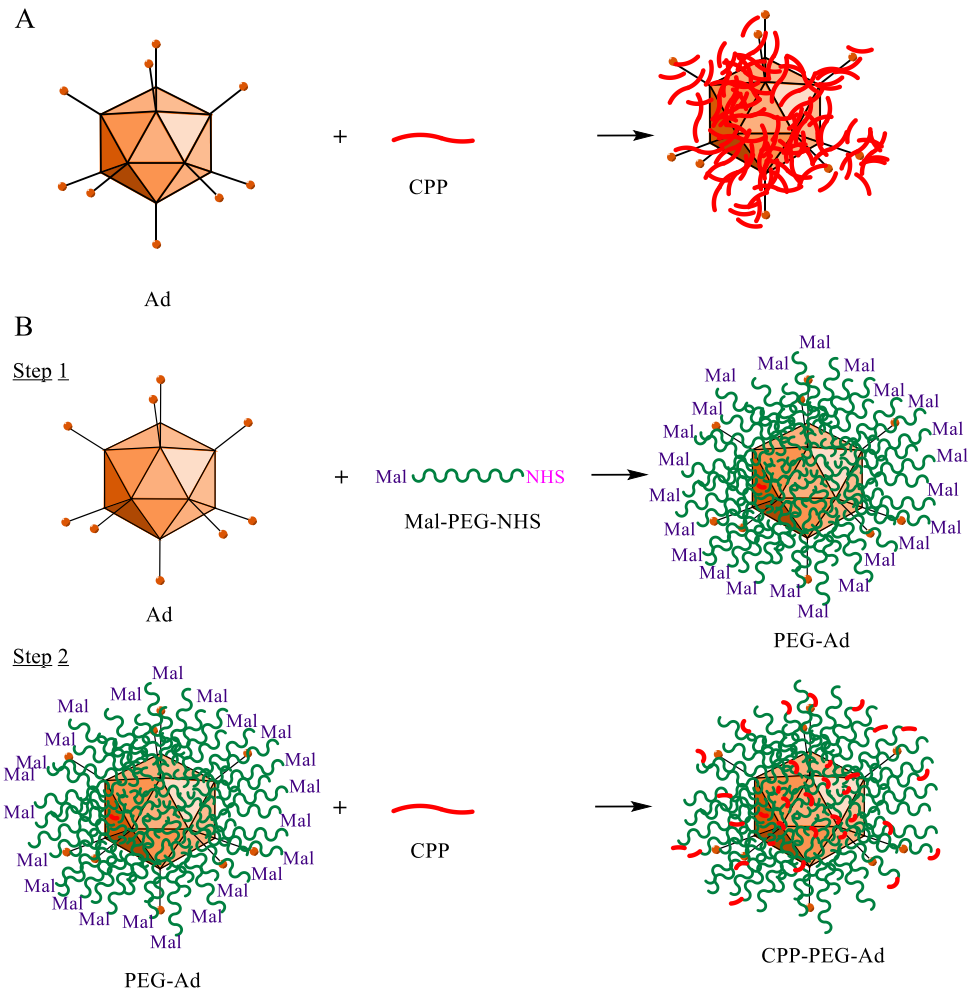

Figure 4.1: Approach for producing CPP/Ad and CPP-PEG-Ad particles. (A) The positively charged CPP electrostatically binds to the negatively charged Ad. (B) In Step 1, the N-hydroxyl succinimidyl ester (NHS) chemical group on the heterobifunctional MAL-PEG-NHS reacts with lysine residues in the fiber and capsid protein of the native adenovirus particle to produce PEGylated virus. In Step 2, the thiol-reactive maleimide (MAL) reacts with the cysteine sulfhydryl group on the CPP to produce CPP-PEG-Ad.

4.2.8. Innate Immune Response to Modified Ad Particles

The degree of the modified Ad to induce an innate immune response was evaluated by measuring the amount of the pro-inflammatory cytokine, interleukin 6 (IL-6), produced by murine macrophage RAW 264.7 cells. The IL-6 secretion in response to Ad, CPP/Ad complexes, and CPP-PEG-Ad particles was measured using ELISA. RAW 264.7 cells (1×10^5 cells/well) were seeded on 12 well plates with 1 ml of medium per well and incubated

for 24 hours prior to treatment with CPP/Ad complexes, CPP-PEG-Ad particles, or unmodified Ad. The cells were treated with the vectors or unmodified Ad for 32 hours at an MOI of 100. IL-6 levels in the medium were then quantified using an OptEIA ELISA kit (BD Biosciences, Sparks, MD) following the manufacturer's protocol.

4.2.9. Humoral Immune Response to Modified Ad Particles

The susceptibility of Ad, CPP/Ad complexes, and CPP-PEG-Ad particles to neutralizing antibodies (NAb) was evaluated by studying the transduction efficiency of particles in neutralizing anti-Ad serum. HEK293 cells in DMEM with 10% FBS were seeded on 12 well plates at 2.5×10^5 cells/well and incubated for 24 hours prior to infection. The following day, unmodified Ad, PEG-Ad or Pen-PEG-Ad was mixed with serial dilutions of anti-Ad serum collected from immunized mice or serum collected from non-immunized mice. After the addition of NAb, each sample was added to the previously seeded HEK293 cells. Transduction efficiency was studied using the Beta-Glo assay and BCA assay described above.

4.2.10. Particle Size and Surface Charge Measurements

The size of Ad, CPP/Ad complexes, and CPP-PEG-Ad particles was measured using a Brookhaven 90Plus dynamic light scattering (DLS) instrument (Brookhaven Instrument, Inc., Holtsville, NY). Samples were diluted to a concentration of 1×10^7 cfu/ml in DMEM (pH 7.4) with and without 10% CS. The samples were allowed to incubate for 5 minutes to 2 hours before taking particle size measurements. The effective hydrodynamic diameter of the vectors was calculated by taking the average diameter of triplicate samples, with six 30 second measurements per sample. The zeta-potential of the vectors and Ad was

measured using a Brookhaven 90Plus ZetaPALS instrument (Brookhaven Instrument, Inc., Holtsville, NY). Samples were diluted in phosphate buffered saline (PBS) (pH 7.4) to a concentration 1×10^7 cfu/ml with a volume of 1,500 µl. Zeta-potential measurements were performed in triplicate with 10 repeated measurements per sample.

4.2.11. Transmission Electron Microscopy

The morphology of Ad and CPP-PEG-Ad particles was visualized using transmission electron microscopy (TEM). Samples were negative stained with 2% phosphotungstic acid, dried, and placed on carbon-formvar grids. TEM micrographs were taken using a JEOL JEM–2100 scanning TEM.

4.2.12. Statistical Analysis

The data reported are the mean of measurements performed on a minimum of three samples (n=3 or n >3). The standard deviation of the measurements is shown as the standard error. Statistically significant measurements were determined using one-way ANOVA (Holm-Sidak method) with *p*-values less than 0.05 being considered significant.

4.3. Results

4.3.1 Transduction Efficiency of Modified Ad Particles

In our previous study, CPP-PEG-Ad particles were produced by varying the type of CPP, the CPP valency, DOP, and PEG molecular weight to optimize the transduction efficiency of the particles on CAR- cells. From the optimized particles, six CPP-PEG-Ad particles were selected to better understand the impact of PEG on transduction efficiency,

immunogenicity, and particle stability. In the first experiment of this study, the transduction efficiency of the CPP-PEG-Ad particles was evaluated on CAR+ cells and compared with CPP/Ad complexes formed by electrostatically binding CPPs to the virus. The effectiveness of the vector was also compared with particles completely lacking CPP, including PEGylated Ad and unmodified Ad. The effects of the PEG molecular weight (5 and 10 kDa) and DOP (50 and 75%) were also investigated.

On CAR+, HEK293 cells, unmodified Ad produced a level of gene expression corresponding to 4,960 RLU/mg cellular protein. The virus alone provided a baseline level of infection to which the other vectors can be compared (Fig. 4.2A). Addition of a CPP that electrostatically associated with the virus (i.e., Tat/Ad and Pen/Ad) did not have a significant effect on the level of infection. As expected, PEGylating Ad reduced the efficiency of the virus by approximately 15% and did not depend on the PEG molecular weight or the DOP. Further modifying the PEGylated virus by conjugating Tat or Pen, however, restored the transduction efficiency of the vector to levels observed from the virus alone. Similar to PEG-Ad, the PEG molecular weight and DOP used to produce the CPP-PEG-Ad did not have a significant effect on the gene delivery efficiency of the vector.

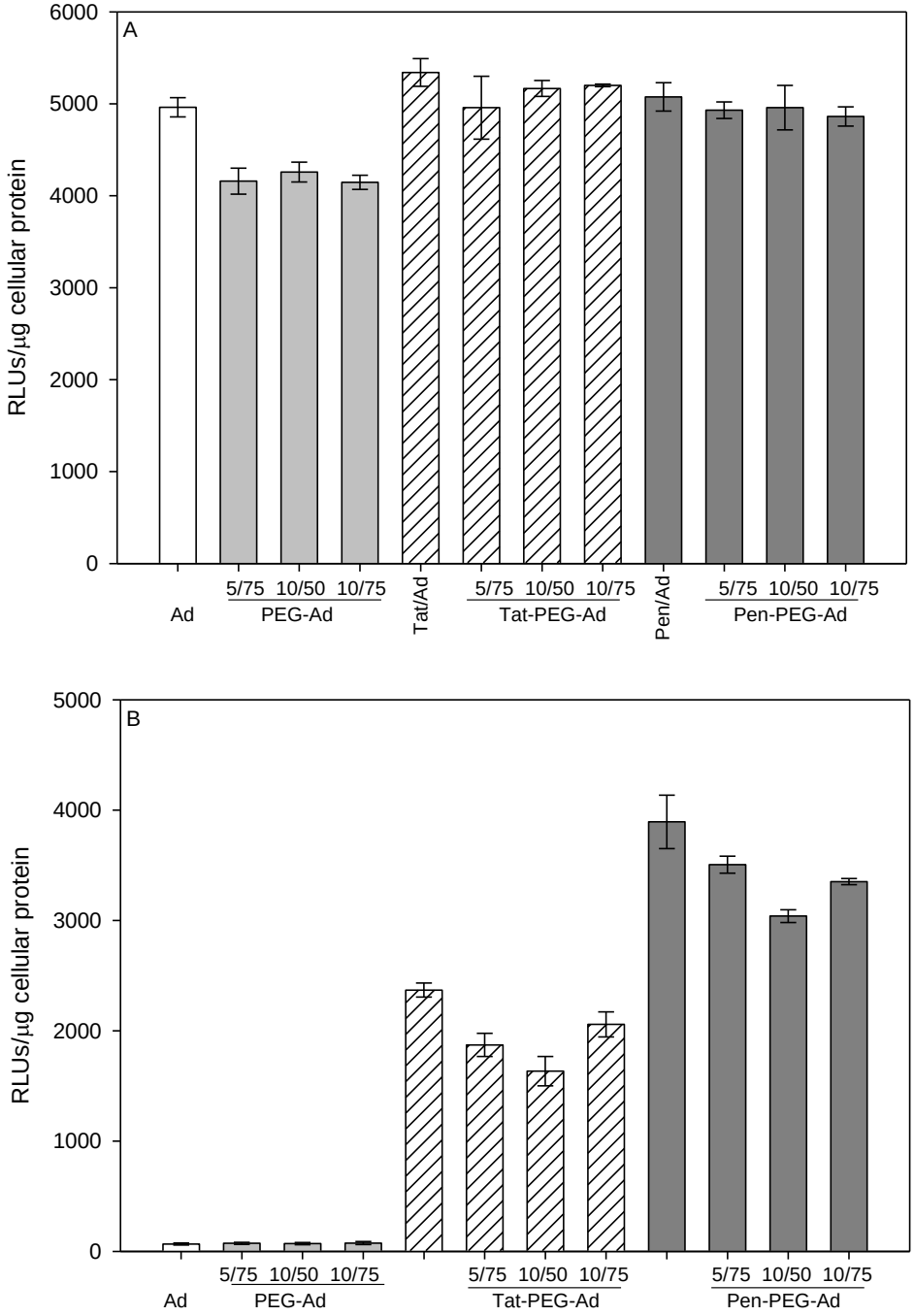

Figure 4.2: Transduction efficiency of unmodified Ad, PEG-Ad, Tat/Ad, Pen/Ad, Tat-PEG-Ad, and Pen-PEG-Ad particles weigh on A) HEK293 and B) NIH/3T3 CPP/Ad and CPP-PEG-Ad particles were produced with CPP valency of 6.25 μg/1×10^6 Ad. DOP of 50 and 75%, and PEG molecular weight of 5 and 10 kDa were used to produce the PEG-Ad and CPP-PEG-Ad. PEG molecular weight and DOP are represented as PEG molecular weight /DOP (5/50).

As demonstrated in our previous work, one benefit of a CPP-modified Ad is the ability to infect CAR- cells that are not normally infected by the native virus. In order to directly determine how well the modified vectors transduce CAR- compared to CAR+ cells, transduction study was performed on NIH/3T3 cells that lack the CAR receptor. In this case, the unmodified virus produced a much lower baseline level of infection (60 RLU/µg cellular protein) since the virus is not able to attach to and infect the CAR- cells. Ad particles modified with electrostatically bound CPPs exhibited improved transduction efficiency (Fig. 4.2B). Pen performed significantly better with 60-fold higher gene expression compared to the virus alone while Tat was only 60% as efficient as Pen. When native Ad was PEGylated the transduction efficiency remained near background levels, similar to those observed from the virus alone. Tethering Tat or Pen to the virus through PEG, however, significantly increased the transduction efficiency to levels comparable to the CPP/Ad electrostatic complexes. Pen-PEG-Ad particles, produced with a 50% DOP and 5 kDa PEG, showed as much as 52-fold higher gene expression, while Tat-PEG-Ad, PEGylated with 5 kDa PEG and 50% DOP, showed as much as 30-fold higher gene expression compared to the unmodified Ad. Also, while not significant, particles produced with 75% DOP exhibited slightly better transduction efficiency than those formed with 50% DOP for both Pen- and Tat-PEG-Ad. Increasing the PEG molecular weight from 5 to 10 kDa while maintaining the DOP at 75 % had no significant impact on transduction efficiency of either CPP-PEG-Ad particles.

4.3.2. Effect of Serum protein on Transduction Efficiency of Modified Ad Particles

Serum protein can have a considerable impact on the transduction activity of vectors due to adsorption. Since gene delivery vectors has to administered in protein-rich environment

in vivo, transduction efficiency evaluation in the previous part of the study was performed in medium containing 10% serum. To better understand the effect of serum protein on infectivity, transduction efficiency study of CPP-PEG-Ad was also carried out on NIH/3T3 in serum-free medium as well as medium containing 10% serum. The result showed that both Tat-PEG-Ad and Pen-PEG-Ad lost ~23% of its activity due to the presence of serum protein (Fig. 4.3).

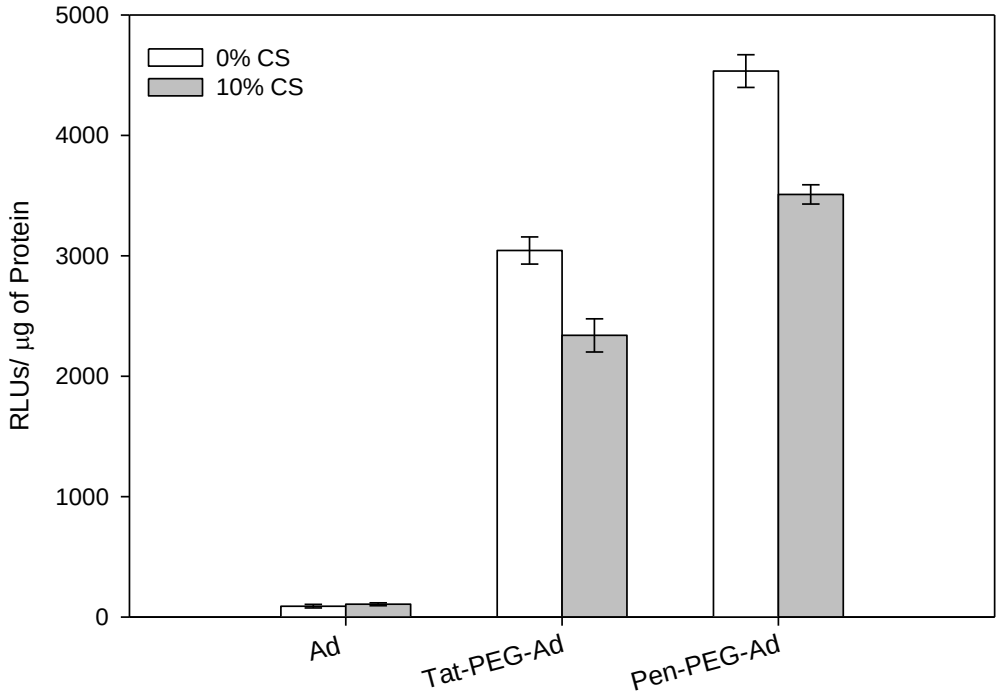

Figure 4.3: Transduction activity of CPP-PEG-Ad on NIH/3T3 cells in the presence and absence of serum while infecting CAR-negative NIH/3T3 cells. Particles were produced with CPP valency of 6.25 µg/1×10^6 Ad. DOP of 75%, and PEG molecular weight of 10 kDa.

4.3.3. Cytotoxicity of Modified Ad Particles

Cytotoxicity of Ad particles modified with CPP was studied on NIH/3T3 cells. Both Tat-PEG-Ad and Pen-PEG-Ad particles had only modest effects on cell growth up to a concentration of 25 μg/10^6 viruses (Fig. 4.4). There was some initial decrease in cell viability with the effect leveling off at about 90% at 25 μg /10^6 viruses. Further increasing the amount of CPP from 25 to 50 μg/10^6 viruses, however, appeared to have a negative impact on cell growth, ultimately reducing the cell viability to 85 % for Tat and 80 % for Pen. The remainder of the study was performed using 6.25 μg/10^6 viruses to minimize the effect of the peptides on cell viability.

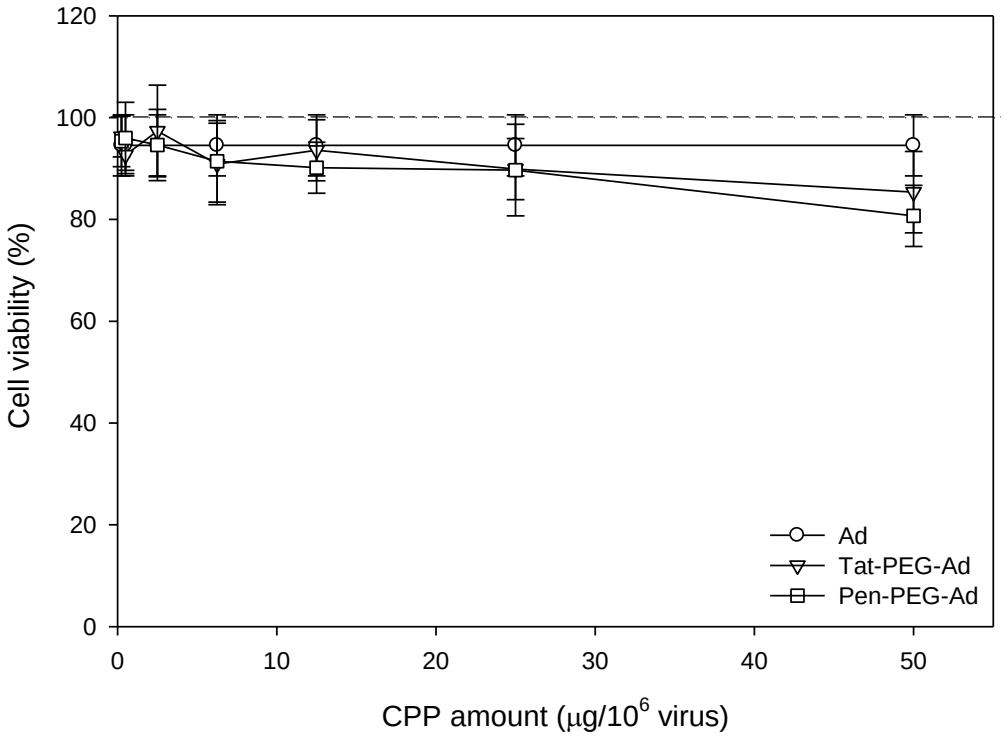

Figure 4.4: Cell viability of NIH/3T3 cells when incubated with CPP-PEG-Ad vectors. CPP concentration (μg/1×10^6 virus) indicates the quantity of CPP used to form the vector prior to infection

4.3.4. Innate Immune Response against Modified Ad Particles

Upon exposure to cells of the immune system, Ad induces the production of pro-inflammatory cytokines such as IL-6, which plays a crucial role in infiltration and differentiation of cells of the immune system and enhances antibody production (Scheller, Chalaris et al. 2011). To understand how modification of Ad with the CPP-PEG conjugate affects this type of immune response, RAW 264.7 macrophage cells were exposed to the particles and IL-6 cytokine production was quantified using an ELISA assay. As shown in Figure 4.5, RAW 264.7 cells secreted 80 pg/ml of IL-6 when exposed to unmodified Ad. Modification of Ad with electrostatically bound CPPs alone reduced to secretion of IL-6 from macrophage cells by 50% and 40% for Tat/Ad and Pen/Ad complexes, respectively. In comparison to the unmodified Ad and CPP/Ad complexes, PEG-Ad and both of the CPP-PEG-Ad particles resulted in significantly lower amounts of IL-6 production. PEGylation of the virus reduced IL-6 production by up to 95%. Addition of the CPP slightly diminished the benefit of PEGylation, but nevertheless, reduced IL-6 production by up to 85%. The study also showed that the type of CPP, DOP, and PEG molecular weight did not significantly affect the amount of IL-6 released.

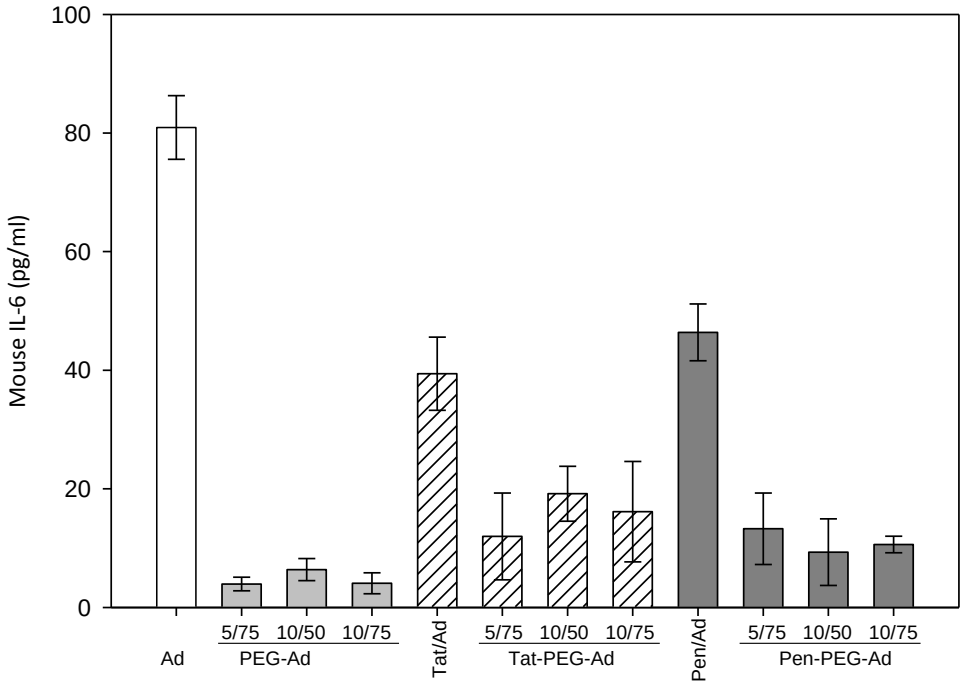

Figure 4.5:.Expression of inflammatory cytokine (IL-6) produced by RAW 264.7 macrophage cells upon exposure to modified and unmodified Ad. PEG-Ad, and CPP-PEG-Ad particles were produced with PEG molecular weight of 5 and 10 kDa and with DOP of 50 and 75%. CPP/Ad and CPP-PEG-Ad particles were produced with CPP amount of 6.25 μg/1×10^6 Ad.

4.3.5. Susceptibility of Modified Ad to Neutralizing Antibodies

Humoral immunity against Ad reduces the gene delivery efficiency of the vector *in vivo*. To determine if the CPP-PEG-Ad vector is also susceptible to this effect, the transduction efficiency of the vector was examined in the presence and absence of serum containing NAb. Figure 4.6 shows the transduction efficiency of unmodified Ad, Pen/Ad, and Pen-PEG-Ad in the presence of NAb serum serially diluted over a range of six orders of magnitude. The infectivity of Ad and Pen/Ad was reduced by up to 87% at the initial Nab concentration. As expected, when the concentration of Nab was reduced, the infectivity of the virus increased. Electrostatically complexing Pen with the virus did not provide any

significant protection to the virus. The Pen-PEG conjugate, however, provided significant protection from inactivation by the NAb serum. Unlike the earlier parts of this study, the PEG molecular weight and DOP had an effect on the level of protection. For example, comparing the PEG molecular weights at a 75% DOP with a 1000-fold dilution of NAb showed that the larger molecular weight provided greater protection; the CPP-PEG-Ad particles produced from 10 kDa PEG and 75% DOP lost 10 % of their activity, while particles produced with 5 kDa PEG at 75% DOP lost 52% of their activity. Likewise, comparing the DOP at a PEG molecular weight of 10 kDa with a 1000-fold dilution NAb showed that the higher DOP also provided greater protection; particles produced from 10 kDa PEG and 50% DOP lost 55% of their activity while particles produced from 10 kDa PEG and 75% DOP only lost 10% of their activity.

4.3.6. Surface Charge of Modified Ad Particles

The surface charge of modified Ad particles determines how the vectors will interact with cells and extracellular components such as serum proteins. The zeta-potential measurements showed that modification of the virus altered the surface charge. The virus alone had a zeta-potential of -21 ± 4 mV (Table 4.1). CPPs electrostatically bound to Ad produced positively charged particle (~12 mV). After PEGylation, however, the vector remained negatively charged at -5 ± 3 mV. Upon addition of CPPs to PEG-Ad, the zeta-potential of the particles became 11 ± 3 mV and 9 ± 2 mV for Pen and Tat, respectively. No significant difference was observed between the surface charge of particles produced with Pen or Tat. Additionally, there was no significant difference between the surface charge of CPP/Ad and CPP-PEG-Ad particles.

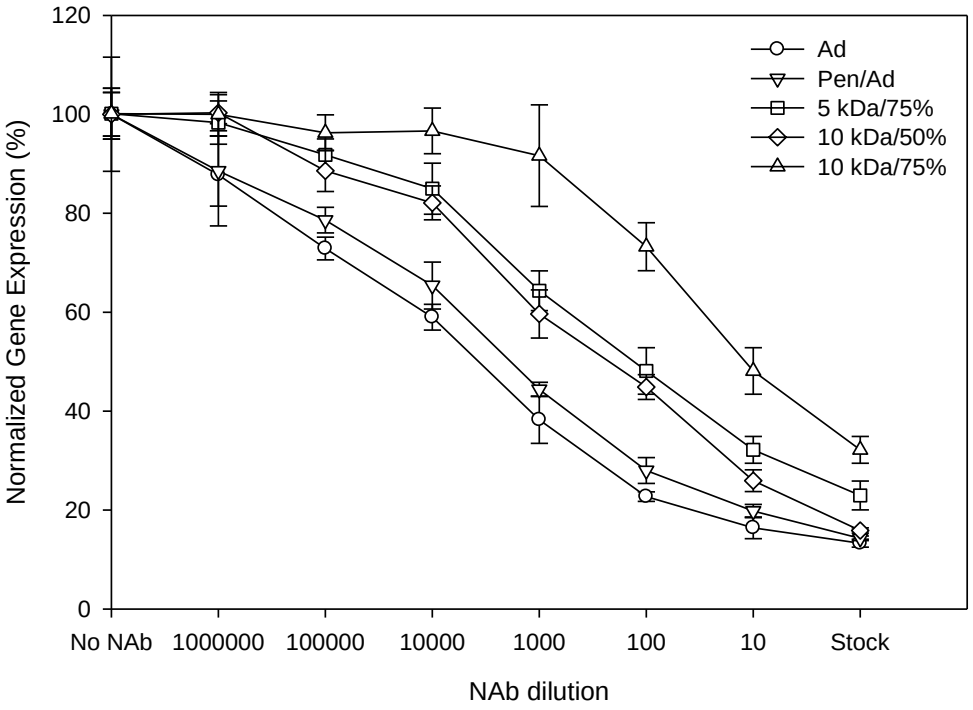

Figure 4.6:.Trasduction of HEK293 cells with unmodified Ad and Pen-PEG-Ad in the presence of neutralizing anti-Ad serum. Pen/Ad and Pen-PEG-Ad particles were produced with 6.25 µg CPP/1×10^6 Ad. The legend indicates the PEG molecular weight/DOP used to produce the Pen-PEG-Ad particles.

4.3.7. Morphology of CPP-PEG-Ad Particles

The structural morphology of Ad and CPP-PEG-Ad, particles were analyzed under TEM. The capsid icosahedral structure was observed for the adenovirus (Fig. 4.7A). A district dense, uniform spherical structure was observed for Pen-PEG-Ad (Fig. 4.7B).6Ad particles were 80 to 100 nm in size while the CPP-PEG-Ad was approximately 240 – 300 nm.

Table 4.1**:** Hydrodynamic diameter and zeta-potential measurements of Ad, CPP/Ad, PEG/Ad, and CPP-PEG-Ad particles measured by DLS. The PEG-Ad and CPP-PEG-Ad particles were formed by PEGylating Ad to 75% DOP using 10 kDa PEG. The amount ofCPP was held constant at 6.25 µg CPP/10^6 Ad

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Vector	Zeta-potential (mV)	Hydrodynamic diameter (nm)
Ad	21 ± 4	112 ± 15
PEG-Ad	5 ± 3	228 ± 48
Tat/Ad	12 ± 4	215 ± 39
Tat-PEG-Ad	11 ± 3	251 ± 51
Pen/Ad	11 ± 2	212 ± 30
Pen-PEG-Ad	9 ± 2	268 ± 42

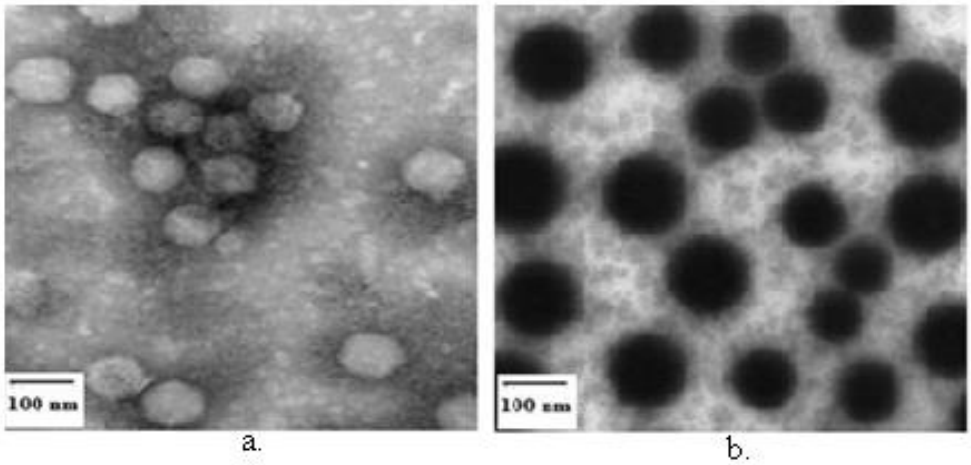

Figure 4.7**:** Transmission electron micrographs of a) Ad and b) Pen-PEG-Ad particles. Samples were negatively stained with phosphotungstic acid and imaged at 30,000X magnificationdiameter and zeta-potential measurements of Ad, CPP/Ad, PEG/Ad, and CPP-PEG-Ad were formed by PEGylating Ad to 75% DOP using 10 kDa PEG. The amount of CPP was held constant at 6.25 µg CPP/10^6 Ad

4.3.8. Stability of Modified Ad Particles

Ultimately, the vector has to be stable under physiological conditions to function effectively. To more closely mimic physiological conditions, the modified Ad particles

were incubated in medium with serum, and the colloidal stability was evaluated by measuring changes in the hydrodynamic diameter and polydispersity (PD) (Fig. 7). The size of the unmodified virus remained stable with time and was monodispersed, with a PD less than 0.2. PEGylation with 75% DOP and 10 kDa PEG initially increased the size of Ad from an average size of 112 to 228 nm (Table 4.1), and the particle size remained stable over the 2 hr incubation in medium with serum. Addition of the CPP, however, had a significant impact on the stability of the particles. Initially, electrostatic modification of the virus with Tat and Pen alone, increased the size of the virus by almost 2-fold (Table 4.1). At short incubation times, the CPP/Ad complexes had a bimodal size distribution, a population close to the size of the virus and a population of larger particles that were likely CPP/Ad complexes. At longer incubation times, larger CPP/Ad complexes or aggregates appeared. The size of complexes increased 6-fold and 5-fold compared to the initial size of the Tat/Ad and Pen/Ad complexes, respectively (Fig. 4.8). Conjugating CPPs to the virus *via* the PEG linker improved the stability of the particles relative to the CPP/Ad complexes. Regardless of the type of CPP, the size of the CPP-PEG-Ad particles increased only moderately as the incubation time increased. Initially the particle size of Tat-PEG-Ad was approximately 251 nm while Pen-PEG-Ad was 268 nm (Table 1). The size increased gradually to 374 nm and 364 nm, respectively, as the incubation time increased. Similarly, as the incubation time of CPP-PEG-Ad particles increased the PD increased as well.

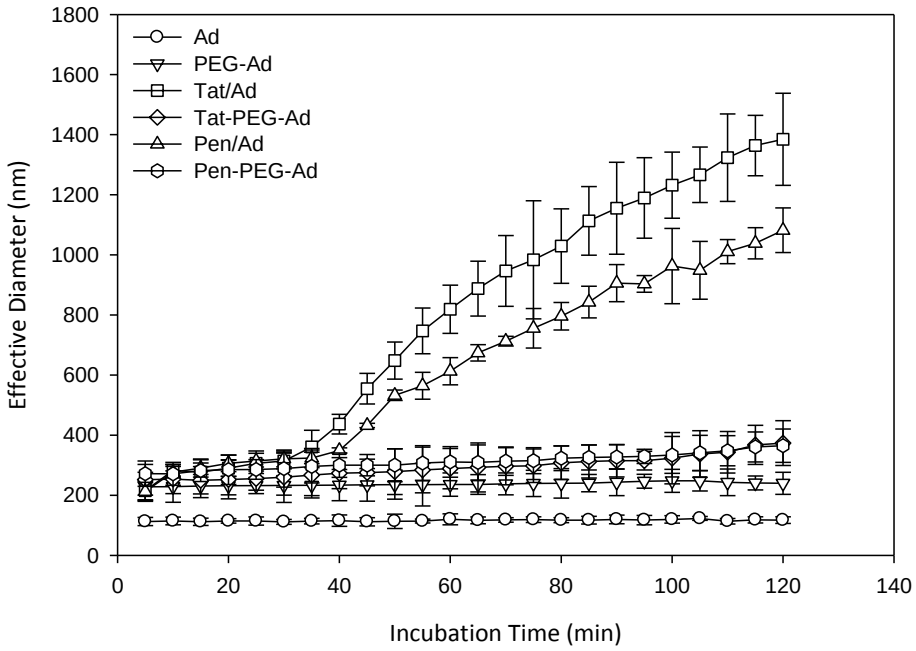

Figure 4.8: The effect of incubation time with serum proteins on the particle size of CPP-PEG-Ad vectors. CPP/Ad, and CPP-PEG-Ad particles were produced with 6.25 µg CPP/1×10^6 Ad. PEG-Ad, and CPP-PEG-Ad particles were produced with a PEG molecular weight of 10 kDa and a DOP of 75%.

4.4. Discussion

In order for Ad-based gene delivery vectors to have a more significant impact on the field of gene therapy, the virus needs to be modified to improve transduction efficiency and reduce immunogenicity (Christ, Lusky et al. 1997, Benihoud, Yeh et al. 1999, Campos and Barry 2007). Studies have shown that PEGylation of Ad improves the susceptibly of the virus to inactivating antibodies and lowers the release of pro-inflammatory cytokines by cells of the immune system (O'Riordan, Lachapelle et al. 1999, Fisher, Stallwood et al. 2001, Croyle, Le et al. 2005, Mok, Palmer et al. 2005). Additionally, our own work and the work of others have demonstrated that CPPs can be used to efficiently augment the translocation of Ad-based vectors into cells that are poorly infected by the native virus

(Gratton, Yu et al. 2003, Kida, Maeda et al. 2006, Lehmusvaara, Rautsi et al. 2006, Youn, Park et al. 2008, Eto, Yoshioka et al. 2009, Park, Doh et al. 2010). By combining these two approaches and tethering CPPs to Ad *via* a PEG linker, vectors have been produced with reduced immunogenicity and improved transduction efficiency (Nigatu, Flynn et al. , Ogawara, Rots et al. 2004, Eto, Gao et al. 2005, Maeda, Kida et al. 2005).

While the advantages of a CPP-PEG-Ad vector have been shown, the effects of the type of CPP, PEG molecular weight and DOP have not been studied extensively. The purpose of the present study was to compare particles produced using two different CPPs and to investigate the effects of PEGylation parameters on the immunogenicity of the vector. Pen and Tat peptides were evaluated since they both showed high transduction efficiency in an earlier study that involved CPP-modified Ad (Nigatu, Flynn et al.). The effect of PEG molecular weight and DOP were investigated by comparing particles produced using 5 or 10 kDa PEG at either 50 or 75 % DOP. The results were compared to electrostatically formed CPP/Ad complexes and unmodified Ad.

Before investigating the immune response to the CPP-PEG-Ad particles, the effects of the type of CPP, DOP, and PEG molecular weight on transduction efficiency were evaluated on both CAR+ and CAR- cells. Ultimately, PEG molecular weight and DOP must be balanced to provide improved immunogenicity while not sacrificing transduction efficiency. If not balanced, PEGylation can compromise the ability of the virus to transform cells by limiting interaction of fiber protein of the virus with the CAR receptor (Maeda, Kida et al. 2005, Mok, Palmer et al. 2005, Wonganan and Croyle 2010). This was observed in the present study when Ad was PEGylated (Fig. 4.2A). The transduction efficiency of PEG-Ad, compared to unmodified Ad, decreased by 15% on CAR+, HEK293

cells. Conjugating CPPs (Tat or Pen) to PEGylated Ad, however, helped restore the transduction efficiency lost as a result of PEGylation. In both cases, neither the PEG molecular weight nor DOP seemed to have an effect on the transduction efficiency under these conditions. Similar results were observed by Eto *et al.* and Maeda *et al.* who showed that PEGylation reduced the transduction efficiency of the virus on CAR+ cells, and addition of an RGD peptide to the PEGylated virus restored the transduction efficiency (Eto, Gao et al. 2005, Maeda, Kida et al. 2005).

The initiating event for infection by Ad is attachment of the fiber protein of the virus to the CAR receptor. Lack of the CAR receptor limits the ability of Ad to efficiently infect CAR- cells. As expected, exposing CAR-, NIH/3T3 cells to Ad alone resulted in very low levels of infection (Fig. 4.2B). Adding CPPs to the PEGylated virus, however, enhanced transduction efficiency by up to 60-fold. In this case, the Pen peptide performed significantly better than Tat on CAR- cells. While Pen-PEG-Ad worked twice as well as Tat-PEG-Ad on CAR- cells, both peptides produced similar levels of transduction on CAR+ cells (Fig. 4.2A). The reason the differences between the two peptides were not observed on the CAR+ cells is likely because of the interactions between the virus and its native receptor, which masks the effect of the peptide.

The performance of the CPPs has been shown previously to depend on how efficiently CPPs associate with the cellular membrane and subsequently induce internalization (Magzoub, Eriksson et al. 2003, Gros, Deshayes et al. 2006). When bound to macromolecules, both Pen and Tat are internalized through energy-dependent endocytosis. The mechanism by which these two CPPs attach to the cell membrane, however, is quite different. Positively charged Tat binds to negatively charged cell surface heparan sulfate

proteoglycans through electrostatic attachment while Pen utilizes both electrostatic binding with cell surface proteoglycans and hydrophobic interactions with the cell membrane (Gros, Deshayes et al. 2006). The combined hydrophobic and electrostatic interactions of Pen with the cell membrane may partly explain why Pen performed better than Tat on CAR- cells that lack the fiber/CAR interactions. The higher translocation efficiency of Pen compared to Tat has been observed by others (Wender, Mitchell et al. 2000, Fischer, Kohler et al. 2004) and discussed in detail (Nigatu, Flynn et al.).

In addition to the type of CPP, DOP also had a minor effect on the transduction efficiency of the vector (Fig. 4.2B). In this particular case, the effect of the DOP was consistent, though not statistically significant. The consistency with which DOP affects transduction efficiency, both in this study and others not reported here, lead us to believe the effect is real. Both Tat-PEG-Ad and Pen-PEG-Ad produced slightly higher transduction efficiency when the DOP was increased from 50 to 75%. The observed increase in transduction efficiency can be attributed to additional CPP conjugated to a more extensively PEGylated virus. In addition to more conjugation sites, a higher DOP forces a change in the PEG conformation, which provides access to sites previously hidden due to a mushroom like conformation PEG usually adopts at lower DOP (Levin, Bishnoi et al. 2006). In contrast to DOP, PEG molecular weight did not have a significant effect on transduction efficiency.

Studies have demonstrated that cytotoxicity of CPPs dramatically change when conjugated to high molecular weight cargoes (Silhol, Tyagi et al. 2002, El-Andaloussi, Jarver et al. 2007). As a result, the modification of Ad with CPPs should produce relatively nontoxic particles. Ad, however, produces its own cytotoxic effects. To understand the combined effects of CPP and Ad on the cytotoxicity of a CPP-PEG-Ad particle, NIH/3T3 cells were

exposed to either Tat-PEG-Ad or Pen-PEG-Ad at various CPP concentrations. The results showed, at low concentrations of CPP (6.25 μg CPP/10^6 Ad), the CPP-PEG-Ad particle not only had a high level of transduction efficiency but also possessed a low level of cytotoxicity that was similar to Ad alone (Fig. 4.4).

One of the main limitations of Ad-based vectors has been immunogenicity (Christ, Lusky et al. 1997, Benihoud, Yeh et al. 1999, Campos and Barry 2007). An innate immune response that follows systemic administration leads to a humoral immune response that can limit repeated use of Ad as a gene delivery vector *in vivo*. Studies have shown that PEGylation weakens the interaction of epitopes of the virus with pattern recognizing receptors on cells of the immune system and reduces a pro-inflammatory signaling cascade induced by these cells (Christ, Lusky et al. 1997, Croyle, Le et al. 2005, Mok, Palmer et al. 2005, Jung, Park et al. 2007, Eto, Yoshioka et al. 2010). The present study has shown that unmodified virus exposed to macrophage cells caused secretion of IL-6 at a high level (Fig. 4.5). Electrostatically coating the virus with Tat or Pen alone reduced the production of IL-6 by RAW 264.7 cells by 50% and 40% compared to unmodified Ad, respectively. This positive effect by CPPs is likely due to an altered mechanism of cellular attachment and entry. The peptides may enable the virus to enter macrophage cells but bypass the epitope-macrophage interaction that promotes cytokine production and phagocytosis. PEGylation of Ad, in contrast, reduced IL-6 secretion by 95%. PEG prevented the viral epitopes from being recognized by the macrophage cells, which limited the cellular response to the virus. Addition of a CPP to produce the CPP-PEG-Ad vector only slightly worsened the response to the PEGylated virus and resulted in IL-6 production that was 85% of the level seen when macrophage cells were exposed to Ad alone. This result might be due to the increase of

cellular uptake of the virus due to the CPP. Over the range tested, PEG molecular weight and DOP had no noticeable effect on the production of cytokines.

Since most patients possess antibodies to Ad from prior exposure, the ability of Ad-based gene vectors to evade neutralizing antibodies is important for gene therapy clinical applications (Wohlfart 1988). Studies have shown PEGylation of Ad reduces the neutralization of the virus by antibodies (O'Riordan, Lachapelle et al. 1999, Mok, Palmer et al. 2005). Similarly, our study showed that CPP-PEG-Ad retained better transduction activity in the presence of NAb compared to Ad (Fig. 4.6). The results also showed that, despite reducing the IL-6 response to the virus, electrostatically bound CPPs did not protect the virus from inactivation by NAb. Unlike transduction efficiency and inflammatory response, resistance to neutralization of the vectors by antibodies was highly dependent on PEG molecular weight and DOP, with the particles retaining greater amounts of their transduction activity at higher PEG molecular weight and DOP. The best protection against the effects of NAb was observed for Pen-PEG-Ad with 75% DOP and 10 kDa PEG. For example, at a 10^3-fold dilution of antibody, the Pen-PEG-Ad particles retained 95% of their activity compared to Ad alone that only retained 38% of its activity.

Physicochemical characterization of CPP/Ad complexes and CPP-PEG-Ad particles showed that their initial sizes (< 300 nm) were appropriate for use *in vivo* (Table 4.1). CPP-PEG-Ad particles were slightly larger than CPP/Ad complexes due mainly to the PEG used to tether the CPP to the virus. There was no noticeable difference in the size or zeta-potential of vectors resulting from the specific peptide between CPP/Ad complexes and CPP-PEG-Ad particles. These results clearly show that the transduction efficiency of the vectors was not exclusively a function of size or surface charge. Instead, the performance

of the vectors was influenced by the CPP and how it affected cellular attachment and internalization of the virus.

In addition to overcoming immunogenicity, the vector must also be stable under physiological conditions. To study this, the stability of CPP/Ad complexes and CPP-PEG-Ad particles were evaluated by incubating the vectors in serum, which mimics physiological conditions. The formation of large aggregates resulting from incubating CPP/Ad complexes in serum indicated that serum proteins adsorbed onto the positively charged particles and significantly affected the hydrodynamic diameter (Fig. 4.7). The effect of serum on CPP-PEG-Ad particles, however, was only moderate, even though both CPP/Ad and CPP-PEG-Ad had similar surface charges (Table 4.1). These results suggest that adsorption of serum protein was not solely dependent on the particle charge. The results also demonstrated that CPP-PEG-Ad particles were much more stable than electrostatically formed CPP/Ad complexes. Despite the relatively modest effect on CPP-PEG-Ad particles, however, serum proteins negatively impacted transduction efficiency. The presence of serum during transduction reduced transduction efficiency by 30% compared to serum-free conditions (Fig. 4.3), which is likely due to the effect of particle size on cellular uptake (Fischer, Bieber et al. 1999, Rejman, Oberle et al. 2004).

4.5. Discussion

Modification of Ad by tethering CPPs to the virus using PEG linkers is an adaptable method that has been shown to improve both the gene delivery efficiency and the immune response associated with an Ad-based vector. The present study showed that the gene delivery efficiency was most strongly affected by the type of CPP and only slightly affected

by the DOP, with PEG molecular weight having no noticeable effect. Both Pen and Tat peptides produced particles capable of infecting CAR+ cells at levels comparable to the native virus. Particles produced with either of the peptides were also able to infect CAR- cells, with Pen enhancing transduction by 60-fold compared to the unmodified virus.

Importantly, while the gene delivery efficiency of the vector was either maintained (on CAR+ cells) or improved (on CAR- cells), the inflammatory immune response and susceptibility of the vector to immune inactivation were significantly enhanced. The inflammatory immune response of the CPP-PEG-Ad vector was reduced by 85% compared to the native virus and was not significantly affected by the type of CPP, PEG molecular weight, or DOP. The resistance to immune inactivation was also improved with the CPP-PEG-Ad retaining 75% of its activity compared to the unmodified virus which only retained 25% activity under similar conditions. In contrast to the inflammatory immune response, resistance to neutralizing antibodies was strongly affected by both PEG molecular weight and DOP.

In conclusion, the type of CPP, PEG molecular weight, and DOP are important factors affecting optimization of a CPP-PEG-Ad vector. Surprisingly, the PEG molecular weight and DOP, within the ranges examined in this study, are not critically important with regard to gene delivery efficiency and the inflammatory immune response but become major factors in protecting the vector from neutralizing antibodies directed against the virus.

CHAPTER V

MODIFICATION OF FIBERLESS ADENOVIRUS WITH MANNOSE-PEG AND PEN-PEG CONJUGATES TO REPLACE THE FUNCTION OF FIBER PROTEIN

5.1. Introduction

Recombinant adenovirus (Ad) has been used widely as a gene delivery vector. Of gene therapy clinical trials conducted thus far, 23% have used Ad-based vectors (Edelstein 2014). Despite wide usage, drawbacks such as broad tropism and immunogenicity are causes for concern. The Ad fiber protein is a contributing factor associated with both of these drawbacks. (Nicklin, Wu et al. 2005, Duffy, Parker et al. 2012). Ad entry into cells is initiated when the knob domain of the fiber protein binds with the ubiquitously found **c**oxsackie virus and adenovirus receptor (CAR), which is responsible for the broad viral tropism (Xia, Henry et al. 1994, Bergelson, Cunningham et al. 1997, Tomko, Xu et al. 1997, Othman, Labelle et al. 2007). Further, the fiber protein presents sites that bind to receptors found on cells of the immune system, which in turn triggers an innate immune response.

While efficient cell binding is a critical first step to transforming a target cell, the fiber/receptor interactions are also associated with some drawbacks. For example, the polyvalency of Ad can lead to agglutination of erythrocytes and severe anemia and

hemoglobinuria (Mori, Yamada et al. 2005, Carlisle, Di et al. 2009).-In addition, Ad fiber protein also has KKTK motifs that facilitate binding to heparin sulfate proteoglycans on the cell surface and promote uptake of the virus by the liver and other off-target cells (Dechecchi, Tamanini et al. 2000, Smith, Idamakanti et al. 2003). These KKTK motifs and binding of the fiber with the broadly found CAR receptor limit the ability of Ad to deliver genes into specific cells. Certain treatments, such as delivery of suicide genes, are therefore difficult to achieve safely and efficiently using Ad. Further, the dependency of the virus on CAR receptor limits the ability to treat certain diseases which require gene delivery to cells that lack CAR receptor such as advanced tumor cells and vascular smooth muscle cells.

In addition to the problems associated with Ad KKTK motifs and the fiber/CAR interactions, the knob portion of the fiber proteins also binds to Fc receptor, scavenger receptor A, and complement receptor-3, which triggers an immune response (Fechner, Haack et al. 1999, Shayakhmetov, Gaggar et al. 2005, Stone, Liu et al. 2007, Xu, Tian et al. 2008, Haisma, Boesjes et al. 2009, Seiradake, Henaff et al. 2009, Khare, Reddy et al. 2012). Preexisting Ad-specific neutralizing antibodies directed against the fiber and capsid proteins also reduce gene delivery efficiency of the vector (GaherySegard, Juillard et al. 1997, Chirmule, Propert et al. 1999, Vogels, Zuijdgeest et al. 2003). Further, the high affinity interaction of fiber and capsid proteins with complements and platelets causes an inflammatory response and leads to cascading effects that promote an adaptive immune response and liver toxicity (Stone, Liu et al. 2007, Xu, Tian et al. 2008, Bradley, Lynch et al. 2012).

Strategies to reduce the drawbacks associated with Ad include modifying the fiber and capsid proteins of the virus with molecules such as liposomes, cell-penetrating peptides

(CPP), polyethylene glycol (PEG), and ligands (O'Riordan, Lachapelle et al. 1999, Natsume, Mizuno et al. 2000, Mizuguchi and Hayakawa 2004, De Geest, Snoeys et al. 2005, Kreppel and Kochanek 2008, Nigatu, Vupputuri et al. 2013). These modifications have been widely applied to reduce immunogenicity of the virus, reduce undesired tropism, enhance gene transfer efficiency, and provide targeted delivery. Another approach to lessening the drawbacks associated with the virus is to genetically alter the fiber protein. Replacing the knob with retrovirus envelope glycoprotein, for example, reduced undesirable interactions between the fiber protein and CAR and other receptors (van Beusechem, van Rijswijk et al. 2000). Genetic replacement of the knob with other Ad serotypes also ablated or altered the viral tropism (Breidenbach, Rein et al. 2004, Nakayama, Both et al. 2006). Together these studies show the role that the fiber protein plays in both the immunogenicity and sometimes-undesirable tropism.

Our unique approach to improve Ad was to completely remove the fiber protein. This approach was expected to reduce the undesired interaction of the virus with cells such as erythrocytes, CAR-expressing cells, and cells of the immune system (Kupgan, Hentges et al. 2014). Removing the fiber protein, however, created the need to replace both its targeting and cell entry functions. The purpose of the present study was to investigate the feasibility of replacing the fiber protein with either (i) a cell-penetrating peptide-polyethylene glycol or (ii) a targeting ligand-PEG conjugate.

The CPP Penetratin (Pen) was used to produce particles by tethering the peptide to fiberless Ad (flAd) *via* a PEG linker to enhance transduction efficiency of the virus in a CAR-independent manner. Alternatively, a mannose (Man) ligand, frequently employed to target cells, was conjugated to flAd using PEG linkers to provide targeted delivery to macrophage

cells known to bear receptors specific to mannose. The transduction efficiency of Pen-PEG-flAd particles was examined on both CAR-positive (CAR+) and CAR-negative (CAR-) cell types, while targeted delivery efficiency of Man-PEG-flAd particles was studied on macrophage cells. The transduction efficiency and targeted delivery of the particles were compared to flAd particles. Additionally, the tendency of flAd-based particles to induce an immunological response was evaluated**.**

5.2. Materials and Methods

5.2.1. Cell Culture

Murine macrophage cell line (RAW 264.7), mouse fibroblast cells (NIH/3T3) and human embryonic kidney cell line (HEK293) were acquired from ATCC (Manassas, VA). RAW 264.7 and HEK293 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) with 10% fetal bovine serum (FBS). The NIH/3T3 cell line was cultured in DMEM, with 10% calf serum (CS) (Mediatech, Inc., Manassas, VA). A fiber-expressing cell line, designated 633, was provided by Dr. Glen Nemerow (Von Seggern, Huang et al. 2000) and cultured in DMEM with 10% calf serum, 1% non-essential amino acids (NEAA) and 1% HEPES. All cell lines were maintained in an incubator with a humidified atmosphere and 5% CO_2 at 37°C.

5.2.2. Fiberless Adenovirus Generation

Previously, a flAd plasmid (pAd5-fl) was generated using homologous recombination to remove the fiber gene from the native Ad plasmid (pAdEasy-1) (Kupgan, Hentges et al. 2014). Since removal of the fiber severely reduced infectivity of the virus, amplification of

flAd required the 633 cell line that stably expresses fiber protein. Transfection of 633 cells with pAd5-fl produced a virus with fiber protein but lacking the fiber gene. Passaging the virus on 633 cells enriched the virus titer. The enriched virus was then used to produce flAd through a final passage on HEK293 cells. The virus was collected and purified using Vivapur Adenopack purification kit following the manufacturer's protocol (Sartorius Stedim Arvada, CO). The virus was quantified by measuring the absorbance of the viral DNA at a wavelength of 260 nm (1 U $\approx 10^{12}$ virus particles/ml) and was stored at -80 ºC.

For the most part, viral particles produced are not able to infect cells since it lacks the fiber protein. If the fiber protein were present, though, or the function of the fiber protein were replaced with some alternate material, then the viral particles are infectious. While generally referred to as infectious particles, it might be better to think about the particles as being *potentially* infectious. Not all native virus or fiberless virus particles, however, are infectious or potentially infectious. Mittereder et al. showed that only about 5% of adenovirus particles are infectious (Mittereder, March et al. 1996). The other 95 % of the virus particles are thought to be inactive due to harsh conditions the viral particles go through during the purification steps. An assumption made in the present study was that just like the native virus, only 5% of fiberless Ad is *potentially* infectious.

5.2.3. Hemagglutination Assay

Human red blood cells collected from individuals with different blood types (O+ and A+) were purchased from Biochemed Pharmacologicals, Inc. (Winchester, VA). An RBC stock solution was prepared by adding 100 µl of RBCs to 10 ml of PBS containing 5% BSA. From the RBC stock solution 50 µl was added to each well of a U-shaped, 96-well plate.

With gentle pipetting, 50 µl of Ad or flAd (2×10^{11} viral particle/ml) were mixed with the RBCs in the plate. As a control 50 µl of PBS containing 5% BSA was used. Plates were incubated for 2 hours at room temperature before being examined for hemagglutination.

5.2.4. Formation of Pen-PEG-flAd Particle

Pen (RQIKIWFQNRRMKWKKC) was synthesized by EZBiolab (Westfield, IN). An additional cysteine residue was added to the C-terminus end of the peptide to facilitate conjugation between the Pen and the PEG. The lyophilized peptide was stored at -20 ºC. Heterobifunctional PEG (maleimide-PEG-NHS) with a molecular weight of 5 kDa was synthesized and purified by Creative PEGWorks (Winston Salem, NC). The Pen-PEG-flAd particle was prepared in two steps. In the first step, the NHS functional group of PEG was reacted with lysine ε-amino groups located on the capsid protein of the virus to form PEG-flAd. The reaction was carried out by adding 4 µg of maleimide-PEG-NHS in HEPES buffer to 1×10^6 flAd in 100 µl HEPES buffer (pH 8.0; 50 mM) while gently vortexing. The reaction proceeded at room temperature for 45 min. In the second step, Pen was added to the PEGylated virus to form Pen-PEG-Ad through a reaction between the thiol-reactive maleimide- group and the C-terminal cysteine residue of the CPP. The Pen-PEG-flAd conjugates were formed by adding 6.25 µg of Pen in 150 µl HEPES (2 µg/µl) to 1×10^6 PEG-flAd particles (Fig. 5.1A). The reaction was carried out at room temperature for 45 min in HEPES buffers (pH 7.0; 20 mM). Excess reactants were removed using a concentrator with a 10 kDa MWCO.

5.2.3. Preparation of Mannose-PEG-COOH Conjugates

Heterobifunctional PEG (NH_2-PEG-COOH) with a molecular weight of 3.4 kDa was obtained from Laysan Bio Inc. (Arab, AL). Mannopyranosylphenyl isothiocyanate (Man-ITC), D-(+)-mannose, lactobionic acid, *n*--(3-dimethylaminopropyl)-*n'*-ethylcarbodiimide (EDC), and *n*-hydroxysuccinimide (NHS) were obtained from (Sigma, St. Louis, MO). Man-ITC was conjugated to heterobifunctional PEG through a thiol-urea reaction following a previously established method (Jiang, Kim et al. 2009). Initially, NH2-PEG-COOH was dissolved in carbonate buffer (pH 9) and added to the Man-ITC dissolved in DMSO. The final mixture was allowed to react at room temperature for 24 hours under gentle stirring. The Man-PEG-COOH was then dialyzed in a Slide-A-Lyzer dialysis cassette with a 3.5 kDa MWCO (Pierce Inc., Rockford, Illinois) against deionized water to remove unreacted Man. After dialysis the Man-PEG-COOH was lyophilized and stored at -80 ºC. The resulting Man-PEG-COOH was characterized by H-NMR (Varian UNITY INOVA 400 NB) using D2O (Cambridge Isotopes, Inc., Tewksbury, MA) as a solvent.

5.2.4. Mannose-PEG-flAd Formation

To form Man-PEG-flAd, lyophilized Man-PEG-COOH, with a PEG:Man ratio 32:1, of was re-suspended in PBS solutions at pH 7.4. With a molar ratio of 1:10:10, Man-PEG-COOH: EDC: NHS, EDC and NHS were added and allowed to incubate for 30 min to convert the carboxylic acid end of the PEG into a succinimidyl ester. The activated PEG solutions were added to Ad a molar ratio of 1×10^7 PEG:flAd and allowed to react with the lysine residues on the flAd surface at room temperature for two hours (Fig. 5.1B). Excess reactants were then removed from the PEGylated vectors using a concentrator with

a 10 kDa MWCO (Sartorius Stedim, Arvada, CO) and the Man-PEG-flAd was then used immediately

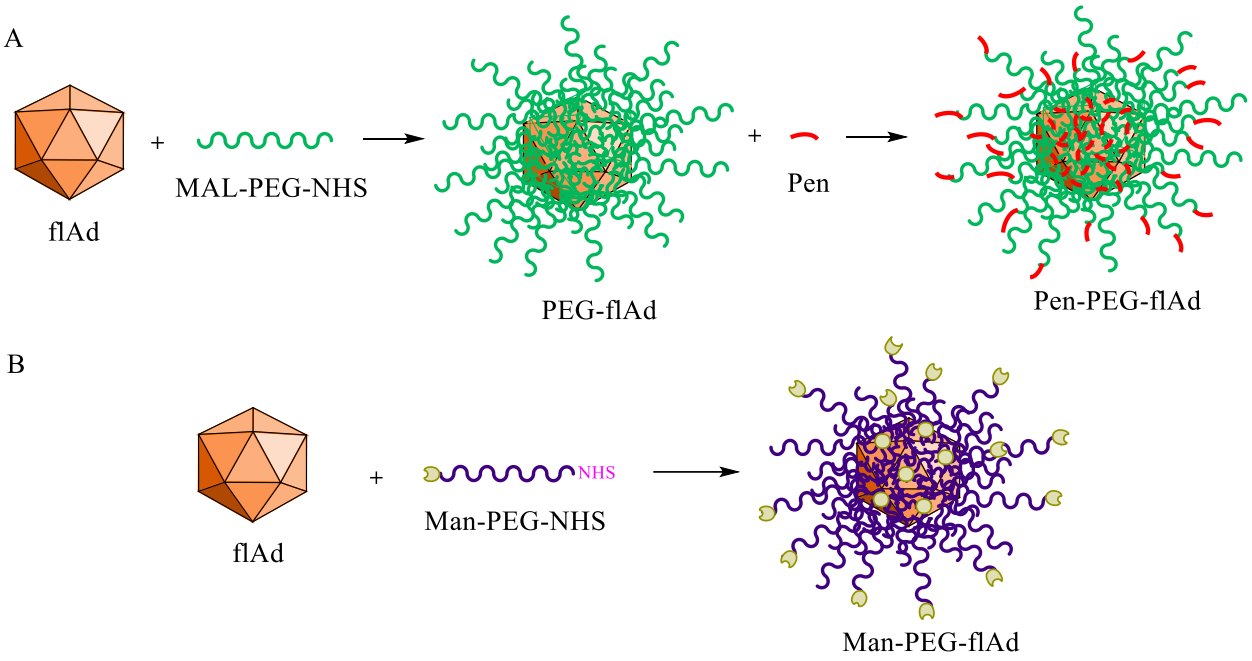

Figure 5.1**:** Approach for producing (A) Man-PEG-flAd and (B) Pen-PEG-flAd particles. (A) Mannose-PEG conjugate was incubated with flAd and reacted with the virus residues form Man-PEG-flAd. (B) First, the N-hydroxyl succinimidyl ester (NHS) chemical group on the heterobifunctional MAL-PEG-NHS reacted with virus capsid lysine residues to produce PEGylated virus. Then, the thiol-reactive maleimide (MAL) reacted with the cysteine sulfhydryl group on the Pen peptide to produce Pen-PEG-Ad.

5.2.5. Pen-PEG-flAd and Mannose-PEG-flAd Characterization

Amount of PEG attached to the capsid of flAd was estimated by using a fluorescamine assay. Briefly, 50 µl of fluorescamine (Sigma, St. Louis, MO), at a concentration of 0.6 mg/ml in acetone, was added in serial dilutions to modified and unmodified virus. The reactions were carried out at room temperature for 15 minutes. Fluorescence measurements were then taken using a PTI fluorometer (Photon Technologies International, Edison, NJ),

with an excitation wavelength of 390 nm and an emission wavelength of 475 nm. Fluorescence measurements were plotted against virus concentration, and the amount of PEG conjugated to the virus was determined by comparing the slopes of the modified and unmodified virus samples (Stocks, Jones et al. 1986).

Following the manufacturer's protocol, an Ellman assay (Pierce Inc., Rockford, IL) was used to estimate the amount of CPP conjugated to PEG-flAd particles by measuring the change in the number of free sulfhydryl groups of unconjugated CPPs. Samples were incubated with Ellman's reagent solution at a ratio of 5:1 in a total volume of 250 μl for 15 minute. The absorbance of each sample was measured at 410 nm. The concentration of cysteine in the sample was estimated using a standard curve produced by measuring the absorbance of known cysteine-HCL standards. Initially, to account for cysteine present within the virus, the amount of cysteine expressed just from the virus was quantified. The amount of conjugated Pen was found by calculating the difference between the total Pen mixed with the PEG-flAd and the amount of cysteine measured from samples containing the Pen-PEG-flAd.

5.2.6. Transduction Efficiency Study

Gene transfer efficacy of Ad, flAd, and Pen-PEG-flAd particles was studied on NIH/3T3 and HEK293 cells. The cells were seeded 24 h before transduction at 2.5×10^5 cells/well on a 12-well plate. The cells were then infected with particles using an MOI of 200 for NIH/3T3 cells and an MOI of 40 for HEK293. The particles were allowed to incubate with the cells for four hours before the media was replaced with fresh media. Reporter gene expression was measured 48 hours after transduction using the Beta-Glo assay (Promega

Inc., Madison, WI) and a Lumat LB9507 luminometer (EG&G, Berthold, Bundoora, Australia). Reporter gene expression was normalized to total cellular protein, which was quantified by a bicinchoninic acid (BCA) protein assay (Pierce Inc.,Rockford, Illinois).

A qualitative transduction study was carried out by staining infected cells with X-gal. Briefly, cells were seeded in a 12-well plate and exposed to viral particles 48 hour prior to the assay. The cells were then fixed with a solution of 4% formaldehyde and 0.5% glutaraldehyde in PBS for 15 min at 4°C followed by twice sets of washing with PBS. A 500 µl of 5% X-gal staining solution was added to the cells and incubated at 37°C for 3 hours. The X-gal staining solution was then removed and replaced with PBS. The cells were then imaged at 10× magnification using an Axiovert 40 CFL microscope (Carl Zeiss International, Jena, Germany).

5.2.7. Competition Assay

RAW 264.7 cells seeded at 2×10^5 cells/well on a 12-well plate were initially incubated with different concentrations of free D-(+)-mannose for 15 and 90 minutes prior infection. The media containing free mannose was replaced with fresh culture media before infecting the cells with flAd, PEG-flAd, or Man-PEG-flAd Four hours after infection, the media was replaced with fresh culture media. Forty eight hours post infection, transduction efficiency of the vectors was evaluated as described above.

5.2.8. Immune Response Study

The effect of the fiber protein and PEGylating on the immune response was evaluated by measuring the amount of pro-inflammatory cytokine IL-6 expression from Raw 264.7 cells

exposed to flAd, Pen-PEG-flAd, and Man-PEG-flAd particles. The IL-6 expression was quantified using OptEIA ELISA kit (BD Biosciences, Sparks, MD) following the manufacturer's protocol. The resulting amount of IL-6 was compared back to the native Ad.

5.2.9. Particle Size and Surface Charge Measurements

A Brookhaven 90Plus dynamic light scattering (DLS) and ZetaPALS instrument (Brookhaven Instrument, Inc., Holtsville, NY) was used to measure the size and zeta-potential of Ad, flAd, Pen-PEG-flAd, and Man-PEG-flAd particles. Samples were prepared by diluting the particles to a concentration of 1×10^7 cfu/ml in DMEM (pH 7.4) with 10% CS. The hydrodynamic diameter of the vectors was calculated by taking the average diameter of triplicate samples, with six 30 second measurements per sample. To measure zeta-potential of the particles, samples were diluted in PBS (pH 7.4) to a concentration 1×10^7 cfu/ml with a volume of 1.5 ml. Zeta-potential measurements were performed in triplicate with 10 repeated measurements per sample.

5.2.10. Statistical Analysis

The data reported are the mean of measurements performed on a minimum of three samples (n=3 or n >3). Error bars indicate the standard deviation of the mean. Statistically significant measurements were determined using one-way ANOVA (Holm-Sidak method) with *p*-values less than 0.05 being considered significant.

5.3. Results

5.3.1. Hemagglutination Assay

The affinity of Ad and flAd for human RBCs was compared using a hemagglutination assay on both A+ and O+ blood types. The results showed that flAd had reduced affinity to RBCs compared to native Ad (Fig. 5.2A). In the control wells, RBCs settled to the bottom of the U-shaped well, seen in the figure as a well-defined circle. In wells containing RBCs and Ad, however, both A+ and O+ RBCs were observed to form a cloudy suspension because of hemagglutination. In contrast, RBCs incubated with flAd settled to the bottom of the U-shaped well, demonstrating that no hemagglutination occurred between flAd and A+ or O+ RBCs.

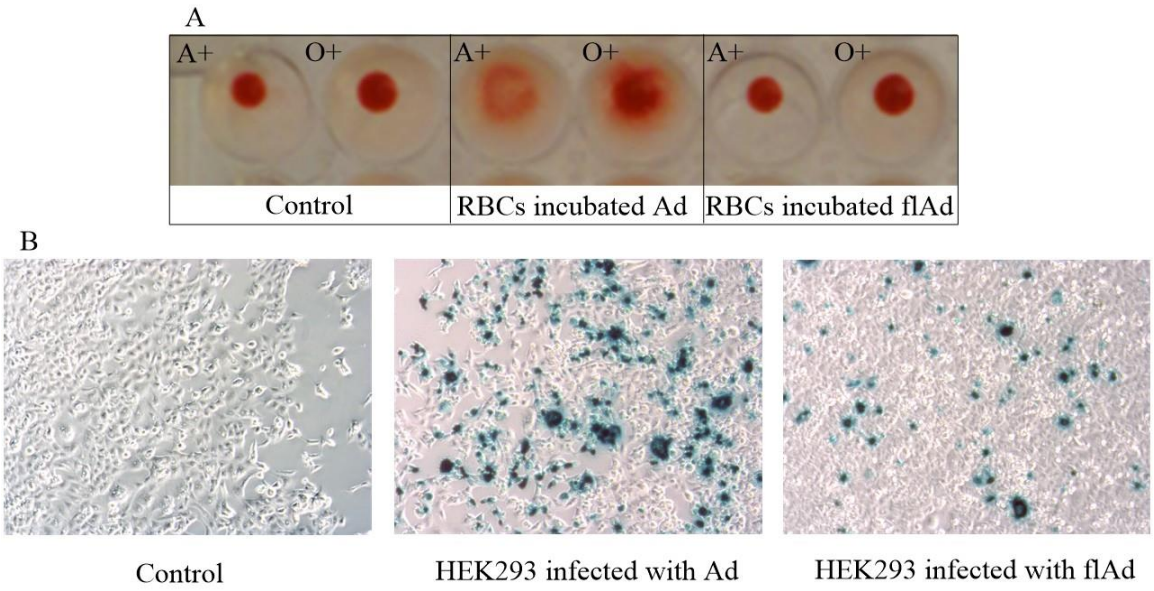

Figure 5.2**:** (A) Agglutination of A+ and O+ human RBCs when incubated with Ad or flAd. (B) X-gal staining of cells transduced with Ad or flAd with an MOI of 0.1.

5.3.2. Characterization of Modified flAd Particles

Man-PEG and Man-PEG-flAd were characterized using proton NMR and a fluorescamine assay. NMR was used initially to characterize the Man-PEG before conjugation to the virus. The NMR results showed that one mannose molecule was present for every 32 PEG chains. To determine how much of the Man-PEG was conjugated to the fiberless virus, a fluorescamine assay was used to quantify how much lysine was present before and after conjugation. By measuring how many fewer lysine sites were available on the surface of flAd, the level of lysine modification or degree of PEGylation of the virus capsid (DOP) was determined. The results of the study showed that 43% of the lysine residues had been PEGylated (i.e., a DOP of 43%). Similarly, a fluorescamine assay was used to estimate the DOP of PEG-flAd, an intermediate product in forming Pen-PEG-flAd. Baded on the results the PEG-flAd particles were PEGylated to a DOP of 55%. An Ellman's assay was then used to quantify how much Pen was conjugated to the PEG-flAd particles to produce the final Pen-PEG-flAd product. The Ellman's assay showed that 72% of added Pen bound to the PEGylated virus.

The size and zeta-potential of Ad, flAd, Pen-PEG-flAd, and Man-PEG-flAd were evaluated using DLS and a zeta-potential analyzer. While the differences were not statistically significant, Ad had a hydrodynamic diameter of 112 nm compared to flAd which had a diameter of 126 nm (Fig. 5.3). Modification of flAd with Pen-PEG or Man-PEG increased the size of the fiberless virus particle to ~ 260 nm and ~ 205 nm, respectively. The surface charge of flAd was approximately -20 mV while unmodified Ad had a charge of approximately -25 mV. The zeta-potential measurement also revealed that

modification of flAd with Pen-PEG increased the charge of the particles to 9 mV while modification with Man-PEG produced near neutral particles (-5 mV).

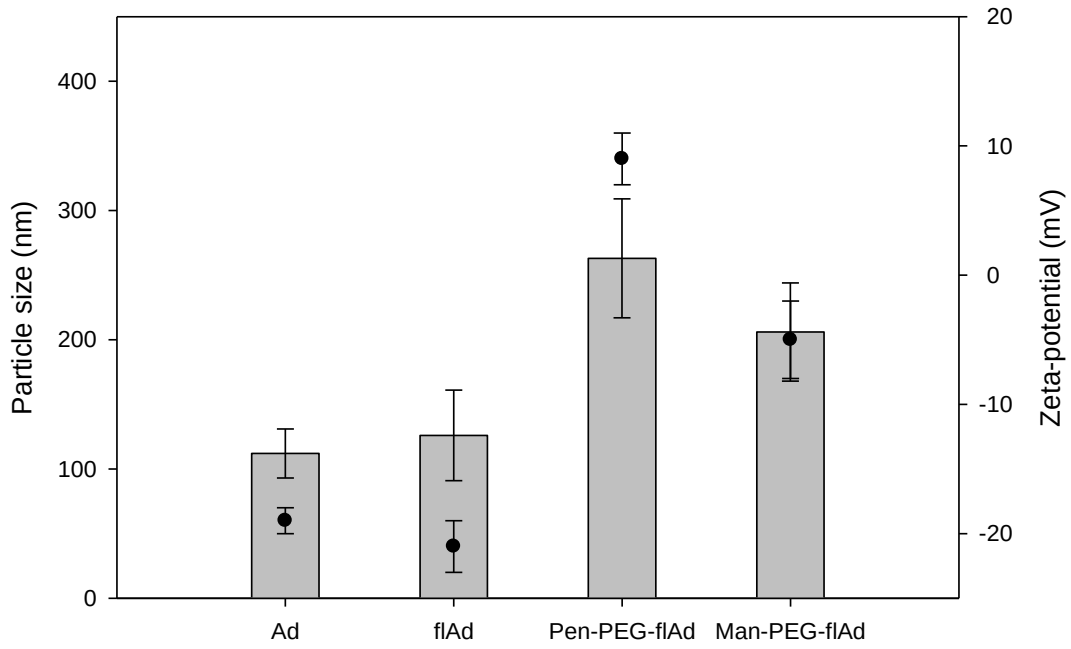

Figure 5.3:.Particle size and zeta-potential characterization of Ad, flAd, Pen-PEG-flAd and Man-PEG-flAd. The Pen-PEG-flAd was produced with 5 kDa PEG with 50% DOP and Pen valency of 1 µg Pen/10^6 flAd. Man-PEG-flAd was produced with 3.4 kDa PEG, 50% DOP, and PEG/Mannose density of 32:1.

5.3.3. Gene Delivery Efficiency of Pen-PEG-FlAd

To evaluate if tethering of Pen to flAd improved gene delivery efficiency, transduction efficiency of Pen-PEG-flAd particles was measured on both CAR+ HEK293 cells and CAR- NIH/3T3 cells. The transduction efficiency of the particles was compared also to the native Ad, flAd, and Pen-PEG-Ad. The result showed that removal of the fiber altered the tropism of the virus. The X-gal staining assay showed that the number of stained cells decreased when cells were incubated with flAd than Ad (Fig. 5.2B). Our gene expression study demonstrated that infectivity of the Ad was reduced by 76% due to the removal of

the fiber in HEK293 cells (Fig. 5.4). Pen improved the transduction efficiency of flAd by 3-fold and restored the 75% of infectivity lost due to the removal of the fiber. Level of transduction of both native Ad and flAd were similar on NIH/3T3 cells (~70RLU/µg cellular protein). Pen-PEG-flAd showed 20-fold higher transduction efficiency of compared to flAd alone but had less 2.5-fold lower efficiency than Pen-PEG-Ad.

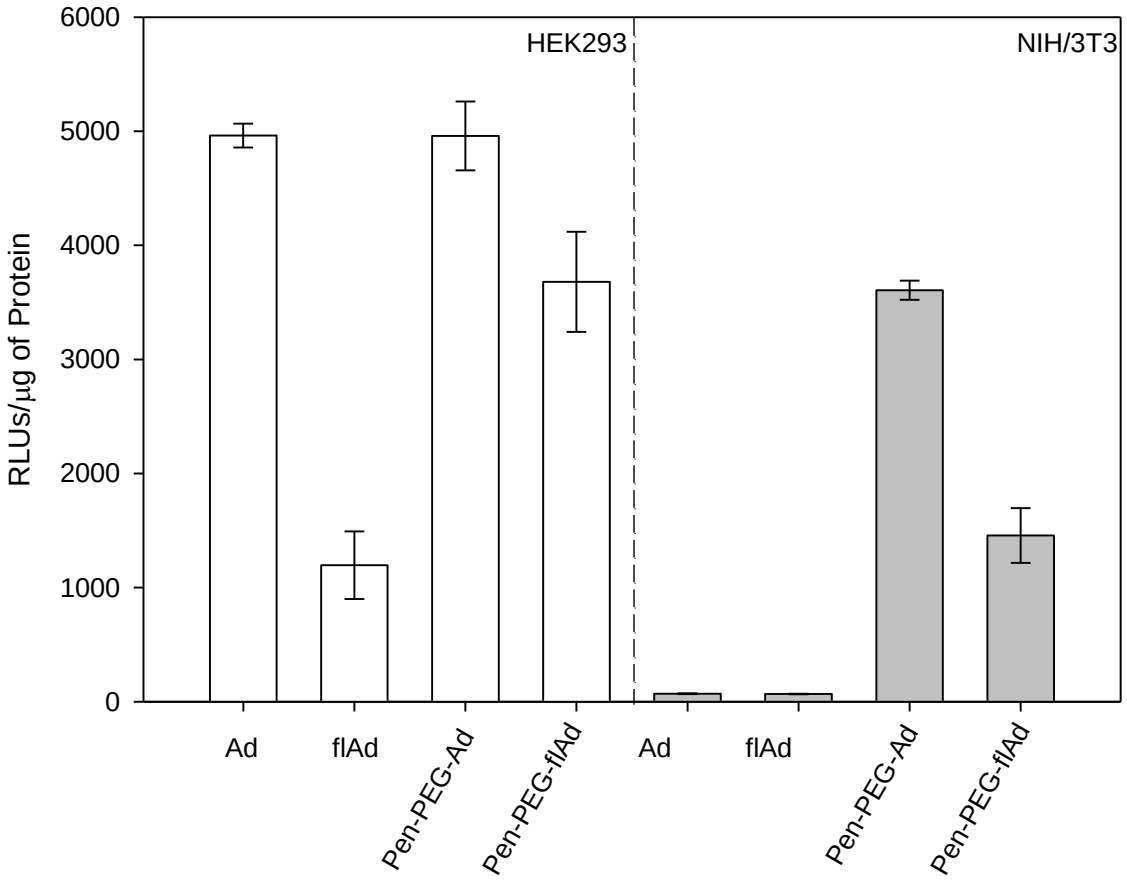

Figure 5.4**:**.Transduction efficiency of study of unmodified Ad, flAd, Pen-PEG-Ad and Pen-PEG-flAd particles in HEK293 and NIH/3T3 cells. The HEK293 and NIH/3T3 cells were transduced by particles with an MOI of 2 and 100 respectively. The Pen-PEG-Ad and Pen-PEG-flAd were produced with 5 kDa PEG, 50% DP, and Pen valency of 1 µg Pen/10^6 flAd.

5.3.4. Targeted Delivery of Mannose-PEG-flAd to Raw 264.7 cells

To evaluate the targeted delivery to Raw 264.7 cells, transduction efficiency study of Man-PEG-flAd was studied. The targeted delivery efficiency of the Man-PEG-flAd was also compared to flAd. The result indicated that the Man-PEG conjugate, which had one mannose molecule per 32 PEG chain, improved targeted delivery of flAd to Raw 264.7 cells by 2.5-fold (Fig. 5.5). There was no significant difference the level of transduction between Ad and Man-PEG-flAd, even though Man-PEG-flAd produced 1.5-fold higher transduction efficiency compared to Ad. In contrast, PEG did not improve gene transfer and showed no significant different to each other.

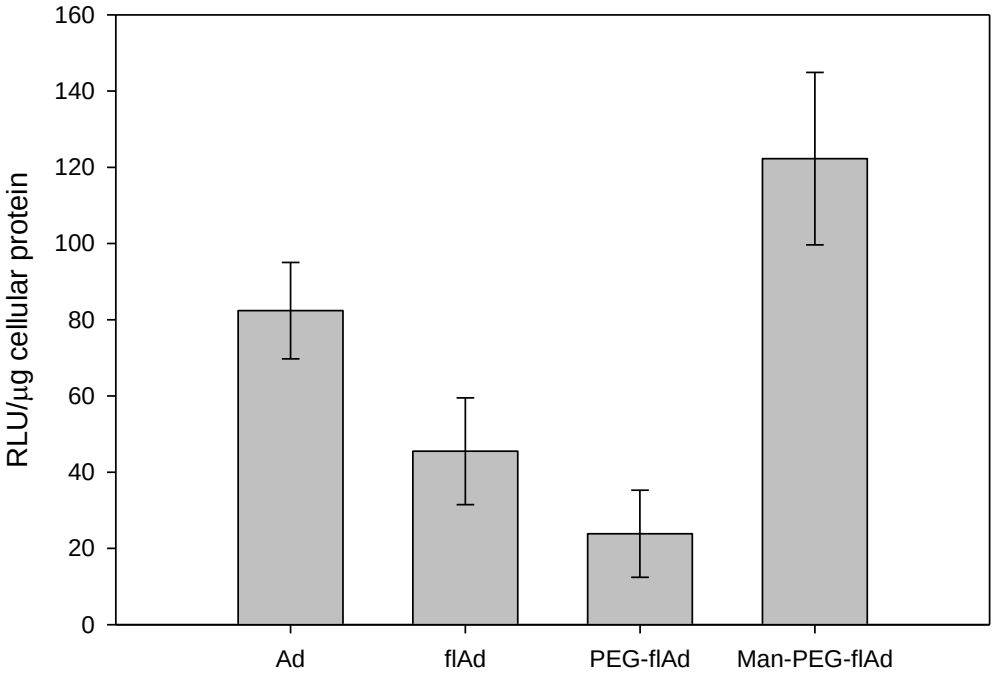

Figure 5.5:.Targeted Delivery of flAd to mannose expressing Raw 264.7 cells. Transduction efficiency of study of unmodified flAd and Mannose-PEG-flAd particles in Raw 264.7 cells. The cells were transduced by particles with an MOI of 200 infective particles. The Mannose-PEG-flAd was produced with a DOP of 50%, PEG molecular weight of 3.4 kDa and PEG/Mannose density of 32:1.

Further, targeted delivery efficiency of Man-PEG-flAd was evaluated by measuring the transduction efficiency on Raw 264.7 cells pre-treated with free mannose. The free mannose competed for the mannose-receptor expressed on the cells and reduce the available binding sites for Man-PEG-flAd. Figure 5.6 shows that Man-PEG-flAd produced high level of gene expression in cells which were not treated with free mannose. Transduction efficiency, however, decreased in cells treated with free Man. Transduction reduced by 10% and 47% when cells were incubated with free mannose for 15 and 90 minutes, respectively.

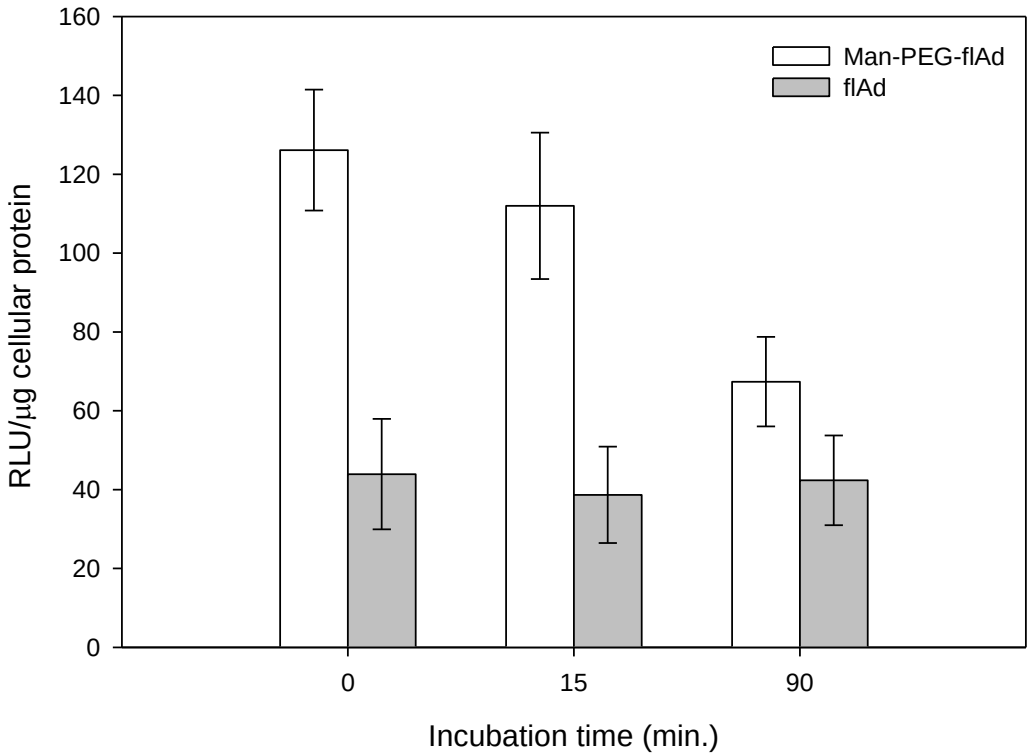

Figure 5.6: Targeted delivery of flAd to Raw 264.7 cells in the presence of computing free mannose. The cells were transduced by particles with an MOI of 200 infective particles. The Man-PEG-flAd was produced with 3.4 kDa PEG, 50% DOP, and PEG/Mannose density of 32:1.

5.3.5. Innate Immune Response Towards Modified flAd

Pro-inflammatory cytokine, IL-6, produced by RAW 264.7 cells when exposed to native Ad, flAd, and modified flAd was measured using ELISA. The study showed that removing the fiber protein only slightly reduced the elicited IL-6 production. RAW 264.7 cells produced 47 pg/ml and 43 pg/ml IL-6 after exposure to native Ad and flAd, respectively (Fig. 5.7). Modification of flAd with Pen-PEG conjugate significantly reduced the cytokine production to ~ 10 pg/ml. Similarly, Man-PEG-flAd induced lower level of IL-6 secretion (6 pg/ml). There, however, was no significant difference between in IL-6 production between cells exposed to Pen-PEG-flAd, and Man-PEG-flAd.

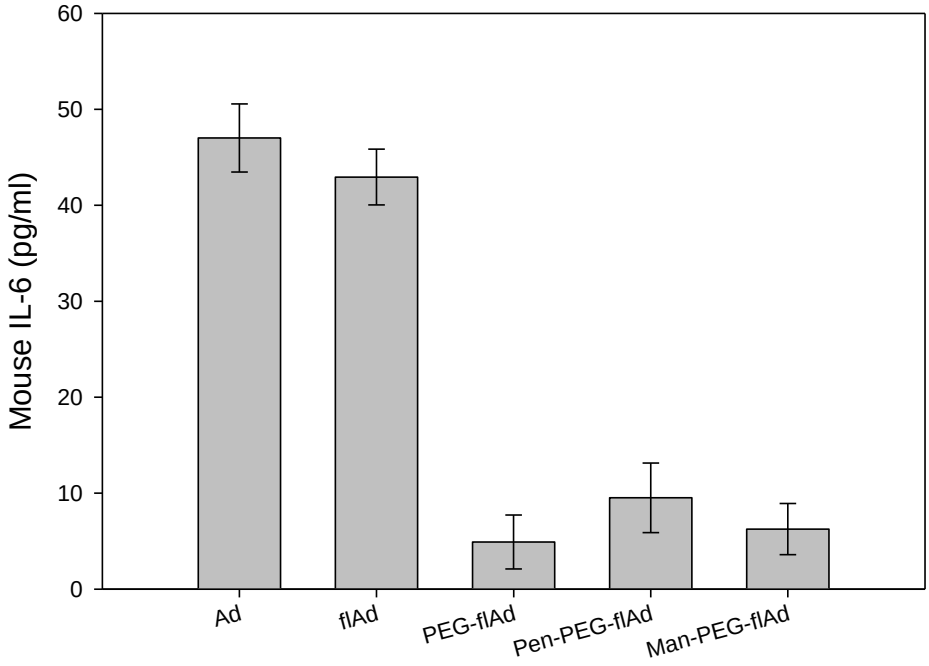

Figure 5.7: IL-6 production by RAW 264.7 due to exposure to unmodified Ad, flAd, and Pen-PEG-flAd particles. The PEG-flAd and Pen-PEG-flAd were produced with 5 kDa PEG, 50% DOP and Pen valency of 1 µg Pen/10^6 flAd. . The Man-PEG-flAd was produced with 3.4 kDa PEG, 50% DOP, and PEG/Mannose density of 32:1.

5.4. Discussions

Ad is one of the most widely used viral vectors in gene therapy clinical trials (Edelstein 2014). The virus, however, has serious limitations, such as promiscuous broad tropism and immunogenicity. These drawbacks are partially attributed to fiber protein of the virus (GaherySegard, Juillard et al. 1997, Chirmule, Propert et al. 1999, Nicklin, Wu et al. 2005, Arnberg 2012, Duffy, Parker et al. 2012). Researchers have made significant efforts to genetically and chemically modify the fiber protein to reduce the drawbacks associated with the virus (Vigne, Dedieu et al. 2003, Wu and Curiel 2008). A more viable approach to completely eliminate the drawbacks of the fiber is to remove the protein and replace its function with biocompatible and non-immunogenic molecules.

Our group previously produced a flAd which was stable at physiological conditions but less robust when compared to the native Ad (Kupgan, Hentges et al. 2014). Our Study on the flAd particles demonstrated that affinity of Ad for erythrocytes was highly reduced by removing the fiber protein (Fig. 5.2). In addition, removing the fiber protein nullified the fiber-receptor interaction which promotes Ad infection. Although removal of the fiber is beneficial in reducing the tropism of the virus, the virus lacks the ability to efficiently deliver therapeutic genes. In the present study, two functionalized vectors were developed by tethering CPPs or ligands to flAd *via* PEG linker to improve the gene delivery efficiency of the particle. The first functionalized vector was produced by coupling Pen to the flAd using a PEG linker in an effort to increase the transduction efficiency of the virus in a receptor independent manner. The second vector was formed by modifying flAd with Man-PEG to target the vector to macrophage cells.

The first step in this study was to ensure that Pen-PEG-flAd and Man-PEG-flAd vectors were physiologically compatible in terms of size and zeta potential. Particle size and zeta-potential studies showed flAd had characteristics similar to Ad. Both Ad and flAd possessed a surface charge of ~-20 mV (Fig. 5.3). There was also no significant difference-in hydrodynamic diameter between the unmodified Ad and flAd. Modification of flAd to produce Pen-PEG-flAd and Man-PEG-flAd significantly increase the particle of the virus from ~120 nm to 263 nm and 206 nm, respectively. The size of vectors were within the desired size that does not cause edema and affect internalization of the particles into cells. The zeta-potential measurements revealed that while Man-PEG-flAd produced less negatively charged particles (-5 mV), Pen-PEG-flAd produced cationic particles with a surface charge of 9 mV. Negative to neutrally charged particles are safer gene delivery vectors compared to positively charged particles (i.e Pen-PEG-flAd). The positive charge can induce electrostatic, non-specific binding that may compromise targeted delivery of the virus.

One of the primary objective of our study was to improve the transduction efficiency of the flAd in a receptor independent manner. Infectivity of Ad is primarily initiated by the binding of fiber protein with CAR receptor. When the fiber is removed, Ad is unable to bind to cells efficiently. In the present study, the infectivity of flAd decreased by 75% compared to native Ad on, CAR+, HEK293 cell (Fig. 5.4). Tethering Pen to flAd *via* a PEG linker restored most of the infectivity lost due to the removal of the fiber and improved gene delivery efficiency by 3-fold compared to flAd alone. Similarly, modification of flAd with Pen-PEG conjugate significantly improved gene delivery by 20-fold in CAR-NIH/3T3 cells. Our group among others have shown that Pen covalently or electrostatically

bound to Ad augment the transduction efficiency of the virus (Gratton, Yu et al. 2003, Lehmusvaara, Rautsi et al. 2006, Nigatu, Vupputuri et al. 2013). Pen peptide binds to cell membrane through hydrophobic interaction and electrostatic interactions to initiate internalization of the particles (Gros, Deshayes et al. 2006).This result is supported by the high surface charge observed for Pen-PEG-flAd particles. These interactions can induce binding and internalization of the particles into a wide range of cells. While these results are encouraging, the non-specific binding of Pen produced an undesired tropism.

Another main criteria of a good gene delivery vector is the ability to deliver the therapeutic gene to a specific site (Campos and Barry 2007). One of the challenges with using native Ad as a gene delivery vector is the broad natural tropism which is mainly governed by the interaction between the fiber protein and CAR. Substituting the fiber protein with Man-PEG conjugates can efficiently target the virus to specific tissues. High expression of mannose-receptor on macrophage cells was used to direct the flAd particles. Modification of flAd with Man-PEG produced similar level of gene transfer to RAW 264.7 compared to native Ad, demonstrating its ability to replace the function of the fiber protein (Fig. 5.5). Our study also showed that free mannose competing for the mannose-receptor affected the transduction efficiency of virus modified with Man-PEG (Fig. 5.6). The free mannose competing for the mannose-receptor, however, did not affect transduction efficiency of flAd. The result support the idea that Man-PEG-flAd was targeting macrophage cells through mannose-receptor.

Although some aspects of functionality of the fiber was replace by Pen and Man, some of the roles the fiber were not replaced. Studies have shown that in addition to mediating binding to the CAR receptor, the Ad fiber protein also modulates intracellular trafficking.

The fiber protein also helps in the rapid escape of the virus to the cell cytosol by acting as a pH sensor that triggers endosomal lytic activity (Miyazawa, Leopold et al. 1999). The present study shows that Man-PEG-flAd and Pen-PEG-flAd produced lower level of transduction efficiency compared to particles produced from native Ad. This may be due to the role that the fiber plays in post-attachment steps involved in infecting cells.

Another concern with Ad-based gene delivery vector is immunogenicity. The fiber and capsid proteins of the virus binds to scavenger receptor A on cells of the immune system and activate inflammatory responses (Haisma, Boesjes et al. 2009, Khare, Reddy et al. 2012). Interaction of Ad epitope to pattern recognizing receptors on macrophages further induces the production of inflammatory cytokines (IL-6) (Zhang, Chirmule et al. 2001, Duffy, Parker et al. 2012). To determine the pro-inflammatory response, macrophage cells were incubated with Pen-PEG-flAd or and Man-PEG-flAd particles. The amount of, IL-6 secreted from the RAW 264.7 cells was measured. The amount of IL-6 produced by RAW 264.7 cells upon exposure to Pen-PEG-flAd and Man-PEG-flAd particles was compared to the IL-6 produced by cells exposed to flAd and Ad (Fig. 5.7). The results showed that cells exposed to flAd produced slightly lower levels of IL-6 than cells exposed to Ad. This may be due to the absence of fiber protein-receptor interaction, which trigger a cascading effect on the innate immune system (Coughlan, Alba et al. 2010). Cells exposed to Pen-PEG-flAd particles, however, produced a much lower amount of IL-6 than cells exposed to flAd or Ad. Other studies have reported similar findings that PEGylating native Ad and tethering CPPs molecules to the virus successfully protected Ad epitopes from pattern recognizing receptors that trigger the innate immune response (Ogawara, Rots et al. 2004, Eto, Gao et al. 2005). These results support removal of the fiber protein combined with

PEGylation of the virus is an effective way to reduce the inflammatory response against Ad.

5.5. Conclusions

Genetic modification of Ad by removing the fiber protein reduced the promiscuous tropism of the virus. The fiberless Ad is, therefore a good form of the virus to develop an Ad-based gene delivery vector. In the present study, two functionalized vectors were developed by tethering Pen or mannose to the flAd by using PEG linkers in an effort to replace the function of the fiber protein of the virus. The results of the study showed that, removing the fiber protein, significantly reduces the infection of CAR+, HEK-293 cells. A Pen-PEG conjugate attached to flAd was able to function in place of the fiber protein, nearly completely restoring the transduction efficiency of the virus. Removing the fiber protein also reduces infection of a RAW264.7 cell line that has only a moderate amount of CAR receptor. A Man-PEG conjugate attached to fiberless adenovirus, however, functions in place of the fiber protein, enabling the virus to target RAW cells through the mannose receptor and thereby restoring the transduction efficiency to levels comparable to the native virus. While transduction and targeted delivery efficiency of the flAd was improved by the functional molecules, the PEG linker played an important role in reducing up to 80% the inflammatory immune response associated with the virus..

CHAPTER VI

SUMMARY

Gene therapy is a promising alternative to the conventional treatment of acquired and inherited diseases. The advancement of gene therapy, however, has been limited due to the lack of a safe and efficient gene delivery vehicle. A gene delivery vector should ultimately overcome intra- and extracellular barriers to deliver a gene to the nucleus of a target cell. Viruses, such as adenovirus, are natural vectors that can overcome the barriers that hinder gene delivery. In fact, viruses are currently used in approximately 75% of gene therapy clinical trials. Viral vectors have high potential as they are extremely efficient and provide stable gene expression. Unfortunately, issues surrounding oncogenicity and immunogenicity of viral vectors have resulted in severe setbacks. Synthetic vectors, in contrast, are relatively safe, yet lack the necessary efficiency.

Although adenovirus vectors are considered safe and have been used widely as gene delivery vectors, major drawbacks such as immunogenicity and promiscuous tropism are significant concerns. The adenovirus infection pathway is initiated by the binding interaction of the fiber proteins of the virus with coxsackievirus and adenovirus receptor (CAR) present on the cell membrane. The ubiquitous presence of this receptor in a wide

range of tissues limits the use of adenovirus for targeted delivery. At the same time, the dependency of the virus on CAR compromises the ability of the virus to act as a gene transfer vector into cells that lack CAR. In addition, the fiber and capsid proteins of the virus can be recognized by cells of the immune system system and induce an immune response that includes the release of pro-inflammatory cytokines, silencing of gene expression, and clearance of the virus from the body. Pre-existing immunity also prevents administration of the vector in patients with prior exposure. Another drawback is the broad and promiscuous tropism of the virus. Because of these numerous drawbacks, clinical applications of Ad-based vectors have been hindered. The main **goal** of this study was to produce a safe and efficient gene delivery Ad-based vector through chemical and genetic modifications and extend our understanding to transition to producing effective synthetic vectors.

In the first study, an Ad-based vector was produced by PEGylating Ad, and then conjugating CPPs to form CPP-PEG-Ad particles. The study compared the effectiveness of four different CPPs: Pen, Tat, Pep1, and pArg. The effects of CPP amount per virus, degree of PEGylation (DOP), and PEG molecular weight on transduction efficiency were studied on CAR- NIH/3T3 cells. . The results of the study showed that:

- CPP-PEG-Ad particles transduced CAR- cells significantly better than unmodified Ad.
- Pen was the most effective CPP and produced an 80-fold improvement in transduction compared to the unmodified virus followed by Tat, pArg and pep1.

- The Pen peptide utilized a combination of electrostatic and hydrophobic interactions with the cell membrane to maximize cellular association while the other CPPs used only electrostatic or hydrophobic interactions but not both.
- Higher degrees of PEGylation, which prompted PEG to adopt a "brush" conformation, resulted in more efficient CPP-PEG-Ad particles because of both better conjugation of CPPs to the PEGylated virus and better exposure of the conjugated CPPs on the surface of the particle.

In conclusions**,** CPP-PEG-Ad particles efficiently deliver genes to cells that Ad alone would not efficiently infect, thereby extending potential gene therapy treatments to a much broader range of cell types and diseases.

In our second study, an Ad-based vector was developed by tethering cell-penetrating peptides (CPP) to Ad *via* PEG linkers. The effects of the type of CPP, DOP, and PEG molecular weight on transduction efficiency on both CAR+ and CAR- cells and immunogenicity were studied to determine how these parameters affect the performance of the vector. The results of the study showed that:

- The present study showed that the gene delivery efficiency was most strongly affected by the type of CPP and only slightly affected by the DOP, with PEG molecular weight having no noticeable effect.
- Both Pen and Tat peptides produced particles capable of infecting CAR+ cells at levels comparable to the native virus.
- While the gene delivery efficiency of the vector was either maintained (on CAR+ cells) or improved (on CAR- cells), the inflammatory immune response and susceptibility of the vector to immune inactivation were significantly enhanced.

- CPP-PEG-Ad particles were no more toxic than Ad alone.
- The inflammatory immune response of the CPP-PEG-Ad vector was reduced by 85% compared to the native virus and was not significantly affected by the type of CPP, PEG molecular weight, or DOP.
- The resistance to immune inactivation was also improved with the CPP-PEG-Ad retaining 95% of its activity compared to the unmodified virus under similar conditions.
- In contrast to the inflammatory immune response, resistance to neutralizing antibodies was strongly affected by both PEG molecular weight and DOP.

In conclusion, the type of CPP, PEG molecular weight, and DOP are important factors affecting optimization of a CPP-PEG-Ad vector. Surprisingly, the PEG molecular weight and DOP, within the ranges examined in this study, are not critically important with regard to gene delivery efficiency and the inflammatory immune response but become major factors in protecting the vector from neutralizing antibodies directed against the virus.

The final part of our study, two conjugates Pen-PEG and Man-PEG were used to replace the function of fiber protein. The vectors were developed (1) by tethering Pen flAd by using PEG linkers in an effort to replace the function of fiber protein in transforming CAR + cells and enhanced transduction efficiency on CAR- cells and (2) by covalently binding Man-PEG conjugate to flAd to enhance targeted delivery of flAd to murine macrophage

- The Pen-PEG-flAd restored most of the transduction efficiency lost in CAR+ cells due to the removal of the fiber protein.
- Pen-PEG-flAd particle were also able to transform CAR- cells by 20-fold higher than flAd alone.

- Covalently binding Man-PEG conjugate to flAd imparted targeted delivery of the virus to macrophage cells that expresses mannose receptor.
- While transduction and targeted delivery efficiency of the flAd was improved by the functional molecules, the PEG linker played an important role in reducing up to 80% the inflammatory immune response associated with the virus.
- The size and surface charge of Man-PEG-flAd particles was within a size range suitable for *in vivo* applications. Pen-PEG-flAd particles, however, were cationic in nature and can electrostatically bind to Physiological proteins.

In conclusion, the study showed the functions of the fiber protein (targeting and cell entry) can be replaced by either a targeting ligand or a CPP.

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APPENDICES

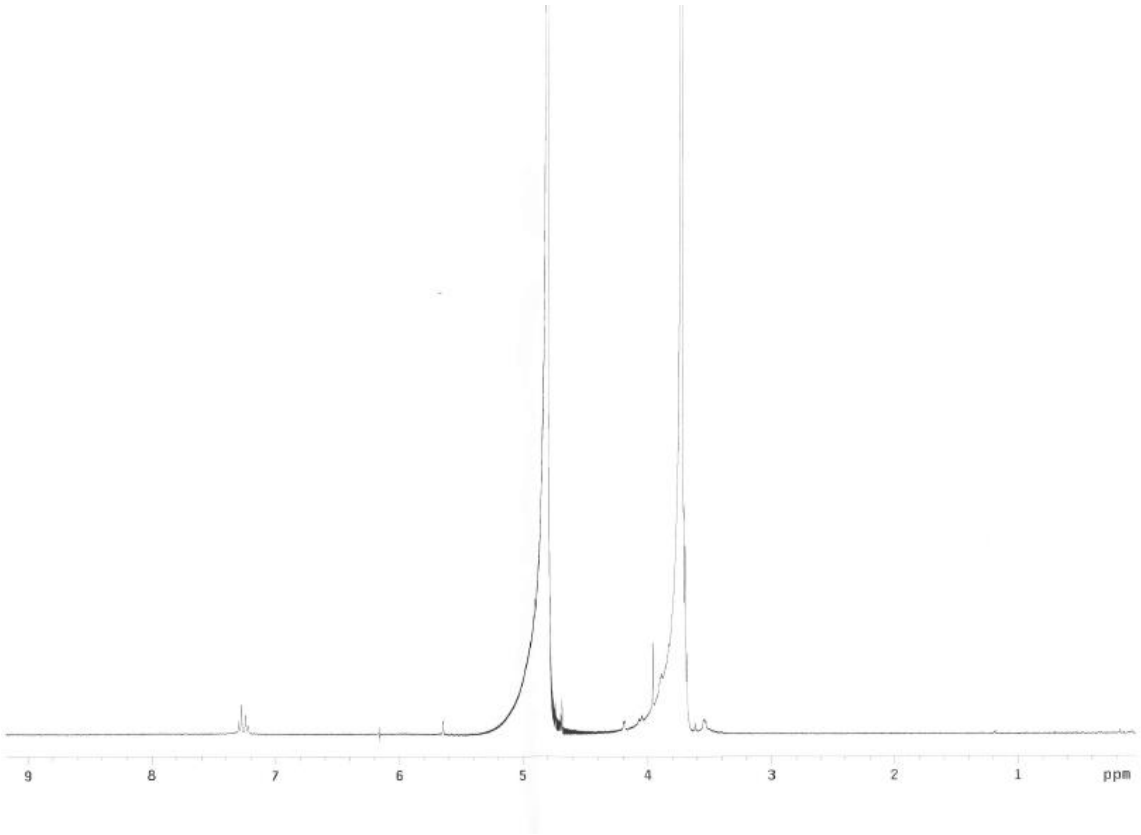

Figure A1. H NMR spectras of Man-PEG. Man-PEG showed peaks of PEG (PEG-H) at 3.8 ppm, and mannose (phenyl-H) at 7.5 ppm.

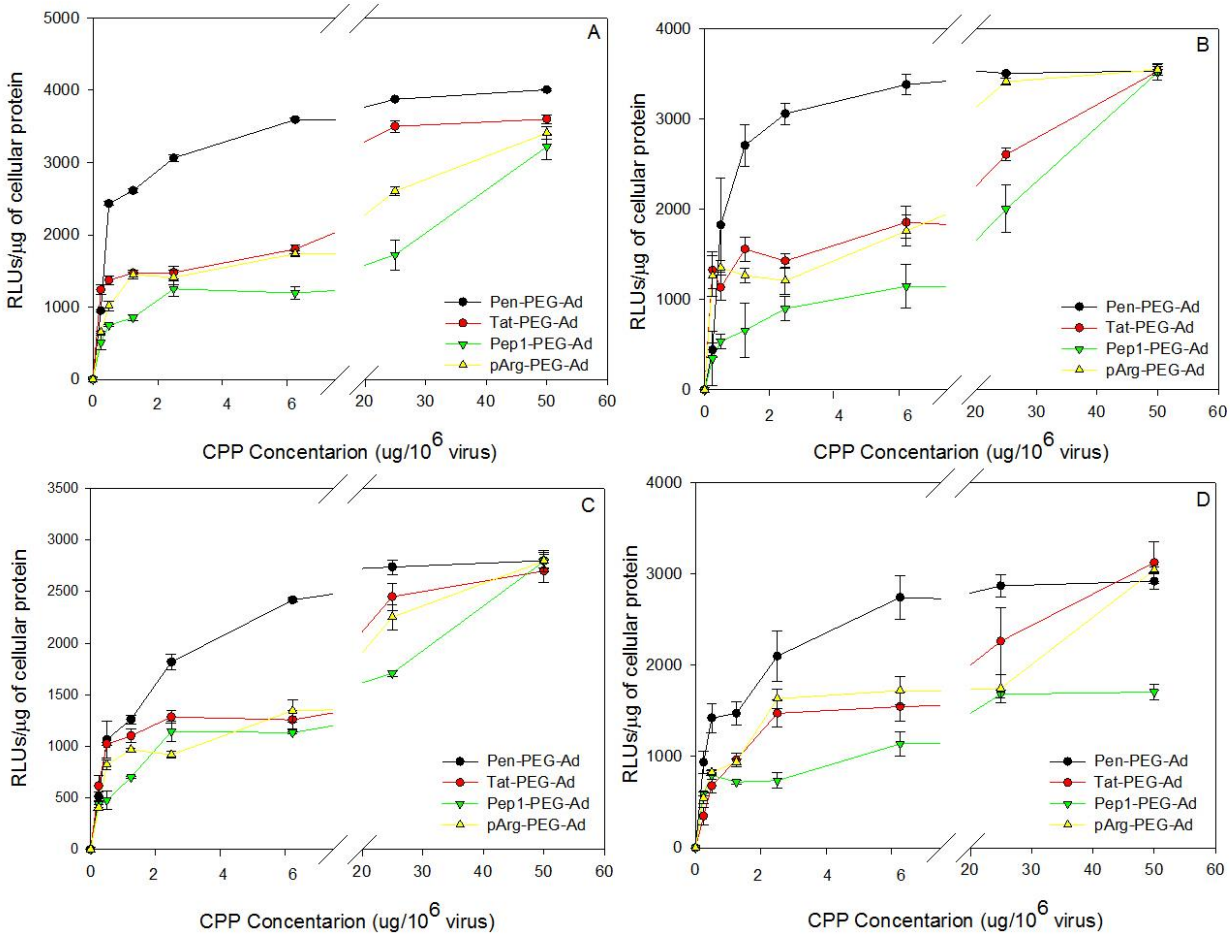

Figure A2**:** Transduction study of CPP-PEG-Ad particles with varied CPP valency and PEG molecular weight of (A) 2 kDa, (B) 3.4 kDa, (C) 5 kDa and (D) 10 kDa to PEG-Ad ratio using a chemiluminescence enzymatic activity assay.

VITA

Adane Nigatu

Candidate for the Degree of

Doctor of Philosophy

Thesis: IMPROVEMENT OF AN ADENOVIRUS-BASED GENE VECTOR: SYNTHETIC MATERIALS THAT REDUCE IMMUNOGENICITY AND FUNCTION IN PLACE OF THE FIBER PROTEIN

Major Field: Chemical Engineering

Biographical:

Adane Nigatu was born in Addis Ababa, Ethiopia. He grew up in Addis Ababa, Ethiopia with two brothers and five sisters.

Education:

Completed the requirements for the Doctor of Philosophy in Chemical Engineering at Oklahoma State University, Stillwater, Oklahoma, USA in May, 2015.

Completed the requirements for the Master of Science in Chemical Engineering at Oklahoma State University, Stillwater, Oklahoma in December 2012

Completed the requirements for the Bachelor of Science in Chemical Engineering at Bahir Dar University, Bahir Dar, Ethiopia in July, 2006

Experience:

Worked as a Graduate Research and Teaching Assistant at the School of Chemical Engineering, Oklahoma State University, Stillwater, Oklahoma from January, 2009 to May, 2015

Professional Memberships:

Active member of AIChE
Active member and officer of ChEGSA
Member of Omega Chi Epsilon